Synthesis, Characterization and Biological Studies of Chromene Derivatives

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ABSTRACT

In this thesis, a series of novel chromene derivatives based on three different moieties of biologically active compounds were synthesized and their antimicrobial and cytotoxic activities were evaluated *in vitro*. The fluorescent 8-amino-10-phenyl-5-hydroxy-2-oxo-4-propyl-2*H*, 10*H*-pyrano [2,3-*f*] chromene-9-carbonitrile derivatives **2.2(a-j**) were synthesized in good yields and with good fluorescence quantum yields (**Φ_F**). The structures were confirmed on the basis of their spectral data and elemental analysis. The antimicrobial activity was investigated and tested against several human pathogens: Gram-positive, Gram-negative bacteria and fungi as well as mycobacterium using agar well diffusion method. Minimum inhibitory concentrations were reported against each pathogen. All compounds showed significantly potent antimicrobial activities against most bacterial strains compared to reference drugs. Due to their structural similarity to reference drugs clorobiocin and novobiocin, docking experiments in the ATP binding pocket of DNA gyrase B enzyme revealed that the compounds mostly have the same binding mode as the reference drugs. Moreover, the cytotoxic activity was also evaluated against four different human cell lines and exhibited more potency than the reference drug. Several new 8-amino-10-phenyl-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-diydro-2*H*,10*H*-pyrano[2,3-*f*] chromene derivatives **3.2(a-g)** were synthesized in good yield. The structures of these derivatives were established on the basis of IR, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and elemental analysis. All new synthesized compounds were evaluated for *in vitro* antimicrobial and cytotoxicity activities. Their antimicrobial activity was investigated and tested with seven human pathogenic Gram-positive and Gram-negative bacteria, four fungi and one mycobacterium, using agar well diffusion method, and minimum inhibitory concentrations were reported. Most of the new tested compounds exhibited significant potent antimicrobial activities against most bacterial strains compared to reference drugs. Some of these new synthesized derivatives exhibited the highest inhibitory activity against *Pseudomonas aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052) compared to reference drugs. On the other hand, three of these new compounds were found to be more effective against *Salmonella typhimurium*. The antibacterial activity of most compounds was found to be comparably active to reference drugs against Gram-positive bacteria, while compound **3.2c** did not show any antimicrobial activity. The cytotoxic activity was also evaluated against four different human carcinoma cell lines and exhibited good cell growth inhibitory activity against HCT-116 cell line. The molecular modeling results showed binding interaction of some new synthesized compounds in the active site of Gyrase B. A variety of novel derivatives of 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4*H*-benzo[*h*]chromene-3-carbonitrile **4.2(a-j**) have been prepared *via* three component reactions. The structures were confirmed on the basis of their spectral data and elemental analysis. Their antimicrobial activity was investigated and tested against four human pathogen Gram-positive and Gram-negative bacteria and four fungi. Some newly prepared compounds exhibited antimicrobial activities against Gram-negative bacteria and fungi species compared to reference drugs. The bathochromic shift of compound **4.2a** was shown in solution when acidified. Moreover, the cytotoxic activity was also evaluated against three different human cell lines. Some tested compounds have good IC_{50} against HCT-116 and MCF-7 cell lines. The molecular modeling results showed binding interaction of some new synthesized compounds in the active site of Gyrase B.

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List of Symbols and Abbreviations

°C	degrees Celsius
^{13}C NMR	carbon-13 nuclear magnetic resonance spectroscopy
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
2D	two dimensional
3D	three dimensional
COSY	correlation spectroscopy
δ NMR	chemical shift in parts per million downfield from a standard
d	doublet
dd	doublet of doublets
m	multiplet
t	triplet
s	singlet
q	quartet
DMF	N, N'-dimethylformamide
g	grams
HSQC	heteronuclear single bond quantum coherence spectroscopy
HMBC	heteronuclear multiple-bond correlation spectroscopy
SAR	structure-activity relationships
CNS	central nervous system
MCR	multi-component reaction
US	ultrasound irradiation
MW	microwave
DMSO	dimethylsulfoxide
EtOH	ethanol
PDB	Protein Data Bank
FT-IR	Fourier Transform Infrared Spectrophotometry

J	coupling constant (in spectroscopy, Hz)
L	litre
min.	minute
mL	millilitre
mmol	mill mole
ppm	parts per million
r	room temperature
Hz	Hertz (s^{-1})
cm^2	square centimeter

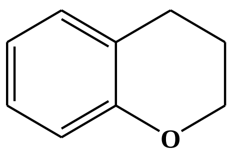
Chapter One: Introduction

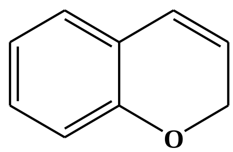
1.1 Scope of present work

This thesis focuses on the synthesis of new bioactive amino chromene compounds based on three selected heterocyclic groups. Chapter Two focuses on the study of the anti-microbial activity of coumarins. Derivatives of 5,7 dihydroxy alkyl coumarin **2.1** were used for the generation of novel derivatives of chromene based coumarins and *in vitro* testing of their antimicrobial and cytotoxic effects, and the fluorescence properties of these compounds is discussed. Chapter Three focuses on the preparation of several novel chromene derivatives based on flavanone moiety and the study their antimicrobial and antitumor properties with docking studies. The work in Chapter Four encompasses synthesis of new chromene derivatives by combination with azo dye and their antimicrobial and antitumor activities and the docking studies are discussed in detail. The purpose of developing these chromene derivatives is to create novel antimicrobial agents that are biologically active especially against infectious diseases, which are one of the most responsible factors for a significant proportion of deaths.

1.2 Chromenes

Heterocyclic chemistry is one of the most complex branches of organic chemistry and heterocyclic compounds are considered the largest family of organic compounds. In fact, these compounds are characterized by their ability to provide structural diversity and have proven to be useful as therapeutic factors.[1] Heterocyclic molecules incorporating oxygen atoms are of special interest and the most important heterocyclic rings containing oxygen are generally tetrahydropyran (6-member). One of the most interesting classes of these materials is the class of chromene compounds. The structure of these molecules includes benzene and pyran rings which are fused together with various levels of saturation. The structures of 1-benzopyrans include various structures such as chromane **1.1**, 2*H*-chromene **1.2** and 4*H*-chromene **1.3** and chromone **1.4** as shown in **Figure 1.1.**


1.1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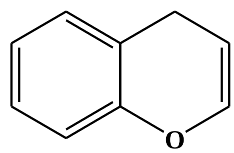
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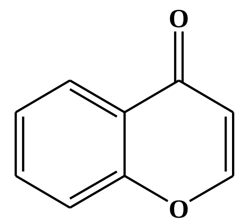
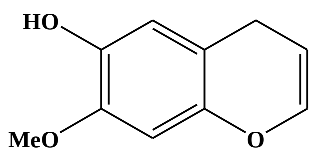

1.4

Figure 1.1: Different structures of 1-benzopyrans compounds.[2, 3]

Chromenes are a group of biologically active molecules occurring extensively in nature with a wide range of molecular modifications.[4] They exhibit a variety of biological effects including anti-microbial[5], anti-viral[6,7], anti-inflammatory[8], anti-oxidants[9], anti-proliferative[10], anti-tumor[11], anti-Alzheimer's and anti-Parkinson disease[12-14], estrogenic[15], antifungal[16], anti-HIV[17] and CNS activity.[18] In addition to their biological activity, certain chromene molecules have been used to produce highly effective fluorescent dyes for synthetic fibers, daylight fluorescent pigments, electro photographic and electroluminescent devices.[19,20]

1.2.1 Naturally occurring chromenes

For thousands of years a number of 4*H*-chromene moieties are found in a large number of naturally occurring compounds such as leaves and stems of plants and sometimes from the roots. Examples of naturally occurring 4*H*-chromene are 7-hydroxy-6-methoxy-4*H*-chromene **1.5** and 6,7-dimethoxy-4*H*-chromene **1.6** which were isolated from the flower of *Wisteria sinensis* that exhibit organoleptic activities **(Figure 1.2)**.



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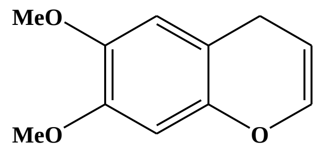


1.6

Figure 1.2: Structure of 7-hydroxy-6-methoxy-4*H*-chromene and 6,7-dimethoxy-4*H*-chromene.[2]

1.2.2 Pharmacological potent and drug like synthetic chromene

A series of synthetic chromene derivatives have significant pharmaceutical potential. Some of them are currently in use as potent drugs and others are in clinical trials. For examples, diethyl 2-(2-amino-6-bromo-3-cyano-4H-chromen-4-yl) malonate **1.7** and 9-amino-7-(3-bromo-4,5-dimethoxyphenyl)-3- methyl - 7*H* - pyrano [2,3-f] quinoxaline-8-carbonitrile **1.8** which are now being developed as anticancer agents. The 2-amino-5, 10b-dihydro-5-oxo-4-phenylpyrano[3,4-c] chromene-1-carbonitrile **1.9** is used as an antibacterial agent.

In addition, 2-amino-4-(3-nitrophenyl)-4*H*-benzo[*h*]chromene-3-carbonitrile **1.10** exhibits anti-rheumatic effect **(Figure 1.3)**.

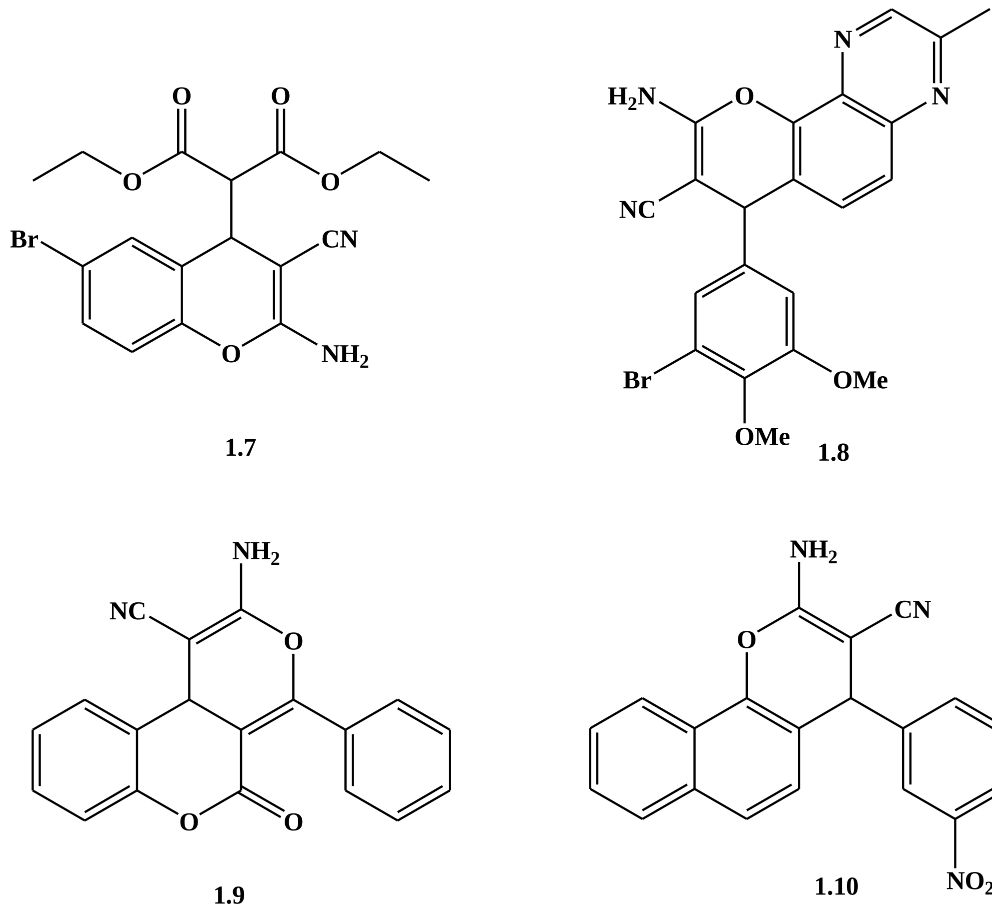


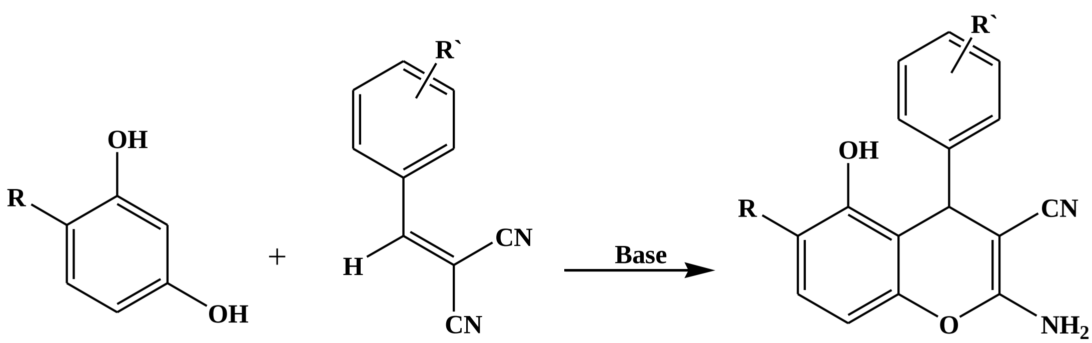
Figure 1.3: Some of the synthetically important drug-like chromene heterocycles.[21-25]

Due to extensive pharmacological applications, chromene heterocycles have considerable interest during the last several years among chemists to develop a useful method to synthesize diverse chromenes with potential interest.

Intense research has been focused on amino-4*H*-chromene and its derivatives have been recognized as one type of privileged medicinal scaffolds due to their unique pharmacological and biological activities. Many structures that contain an amino-4*H*-chromene moiety exhibit excellent biological effects.[26]

1.2.3 General methods for the synthesis of 4*H*-chromenes

In recent years, the multi-component reaction (MCR) has been used to synthesize 4*H*-chromene and its derivatives. In general, 4*H*-chromenes are prepared by condensation of activated phenol, various aldehydes and malononitrile **1.11** in the presence of organic bases. The reaction goes through Knoevenagel condensation product from aromatic aldehydes and malononitrile **1.11**. Finally, the reaction followed by Michael addition involving active methylene compound **1.12** and Knoevenagel product **1.13** to afford 4*H*-chromene **1.14** as shown in the **(Scheme1.1)**.

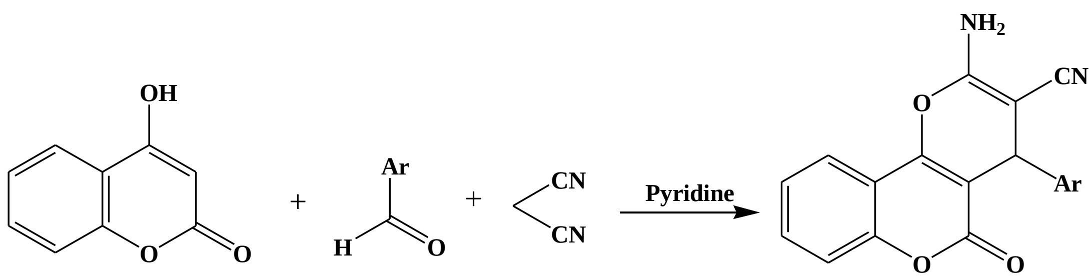


1.12 **1.13** **1.14**

Scheme 1.1: General protocol for synthesis of amino-4*H*-chromene molecules.[27]

Recently, numerous reports are cited in literature in which the various catalysts have been employed for synthesis of amino-4*H*-chromene derivatives.

In 1962, novel 2-aminopyrano [3, 2-c] benzopyran derivatives were prepared by reaction of 4-hydroxycoumarin **1.15** with malononitrile **1.11** and aromatic aldehyde **1.16** in pyridine as a solvent to give desired derivative **1.17 (Scheme 1.2)**.

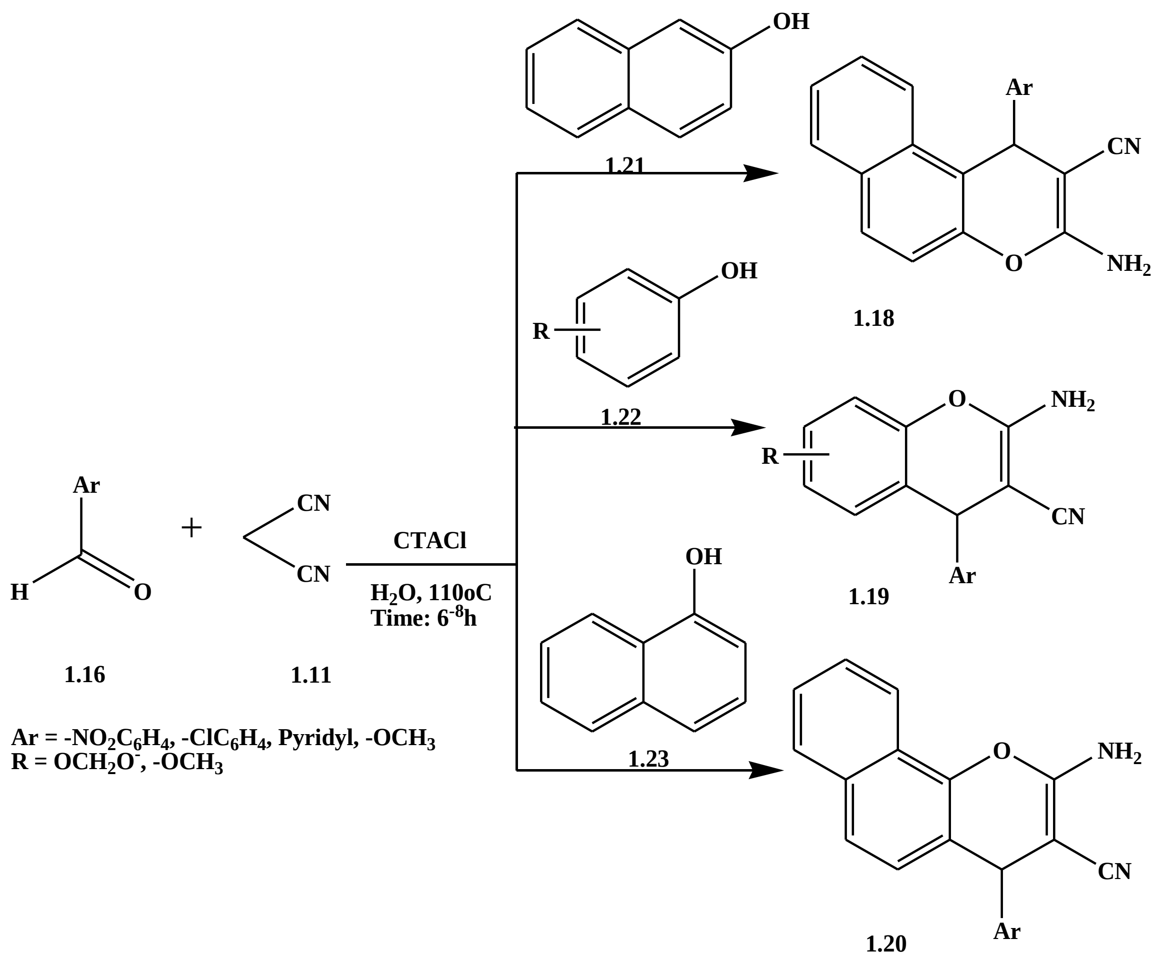


1.15 **1.16** **1.11** **1.17**

Ar = C_6H_5

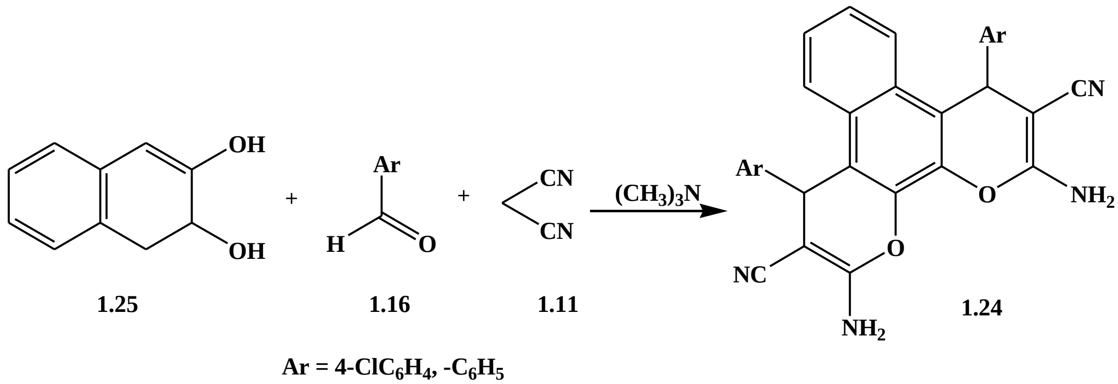
Scheme 1.2: Synthesis of 2-amino-3-cyano-4*H*-chromenes by Wiener and co-workers.[28]

Ballini *et al*. reported the synthesis of 4-aryl-4*H*-chromene heterocycles (**1.18**, **1.19**, **1.20)** via three-component reaction in 2001. The synthetic pathway included condensation between substituted aryl aldehyde **1.16**, malononitrile **1.11** and substituted phenols (**21, 22, 23)** in the presence of cetyltrimethylammonium chloride (CTACl) as catalyst in water under reflux **(Scheme 1.3)**.



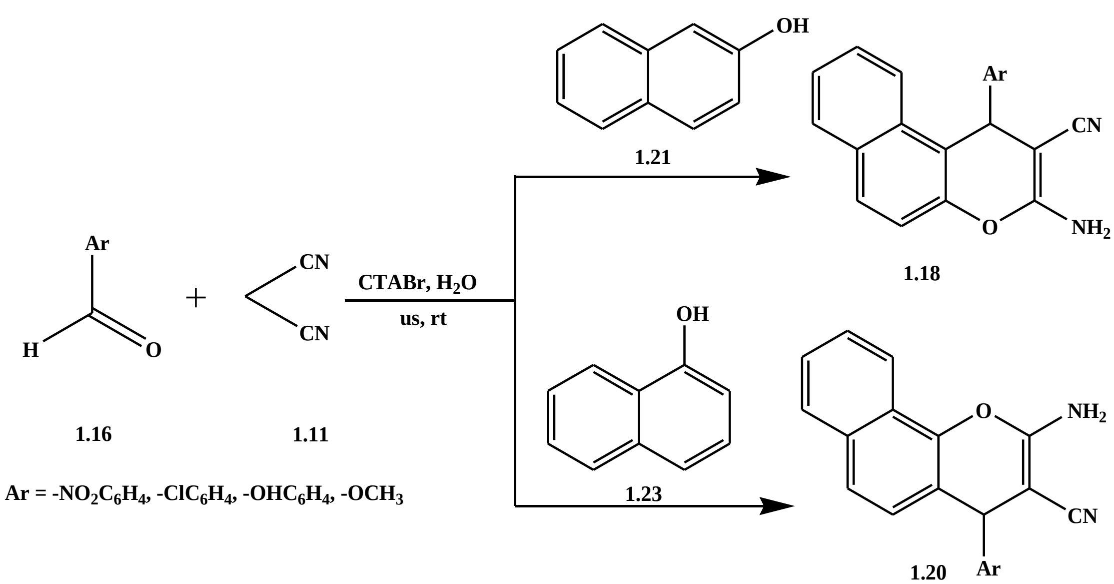
Scheme 1.3: CTACl catalyzed one-pot synthesis of 4-aryl-4*H*-chromenes.[29]

In 2002, Shestopalov *et. al.* reported the synthesis of substituted 2-amino-4*H*-chromenes **1.24** by three component reaction of naphthalenediol **1.25**, aromatic aldehyde **1.16** and malononitrile **1.11** in the presence of trimethylamine as a catalyst in ethanolic solution **(Scheme 1.4)**.



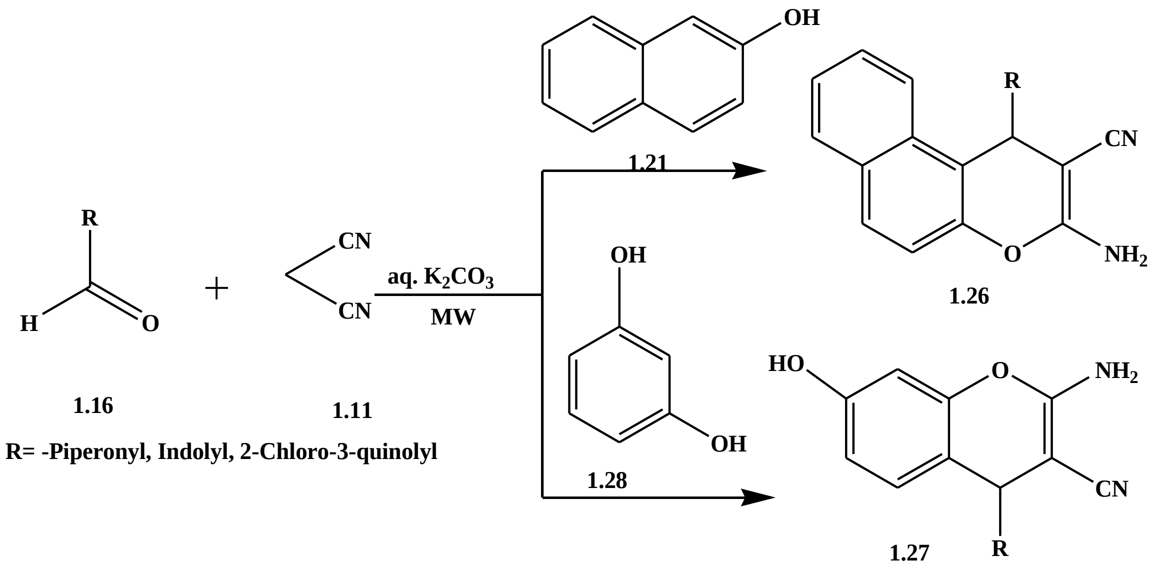
Scheme 1.4: Synthesis of substitutes 2-amino-4*H*-chromenes in triethyl amine.[30]

Novel 2-amino-4*H*-benzo[*h*]chromenes **1.18**, **1.20** were synthesized by multi-component reaction in 2004. The synthetic pathway included condensation between malononitrile **1.11**, aromatic aldehyde **1.16** and 1- and 2-naphthols (**1.23**, **1.21**) and using cetyltrimethyl ammonium bromide (CTABr) as a catalyst in water medium under ultrasonic irradiation **(Scheme 1.5)**.



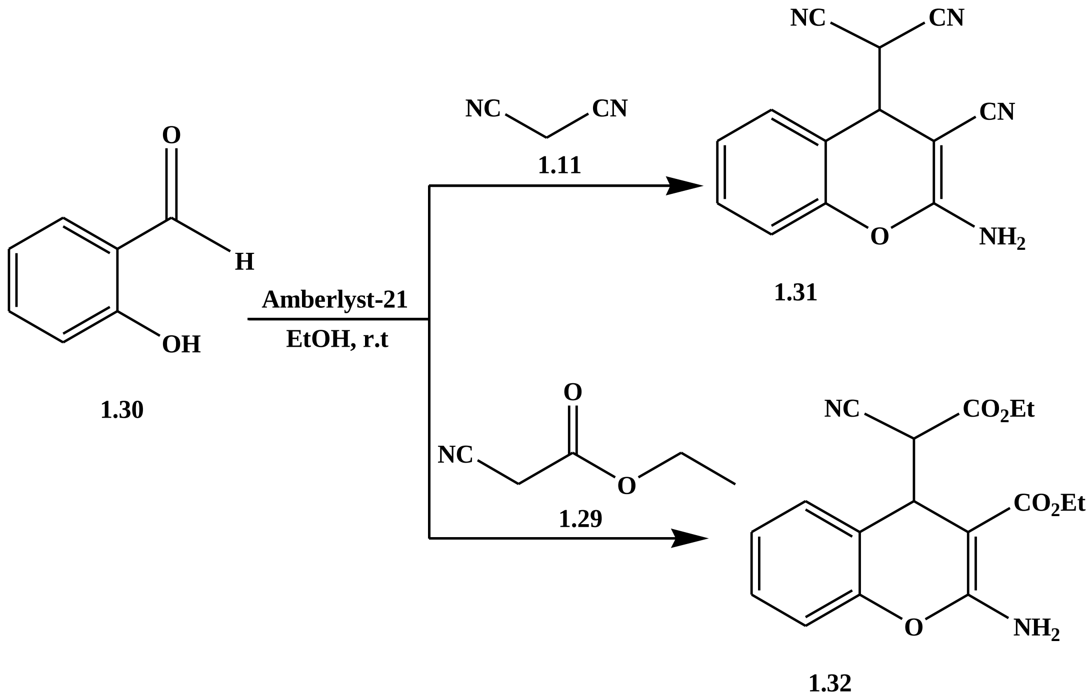
Scheme 1.5: Synthesis of 2-amino-4*H*-benzo[e]chromenes using CTABr under US.[31]

In 2005, two novel 2-amino-3-cyano-4*H*-benzo[*h*]chromenes (**1.26**, **1.27**) were prepared by reacting malononitrile **1.11** with aryl aldehydes **1.16** and 2-naphthol **1.21** or resorcinol **1.28** under microwave irradiation (MW) in K_2CO_3 as a catalyst **(Scheme 1.6)**. Most of synthesized derivatives have *in vitro* antibacterial activity.[32]



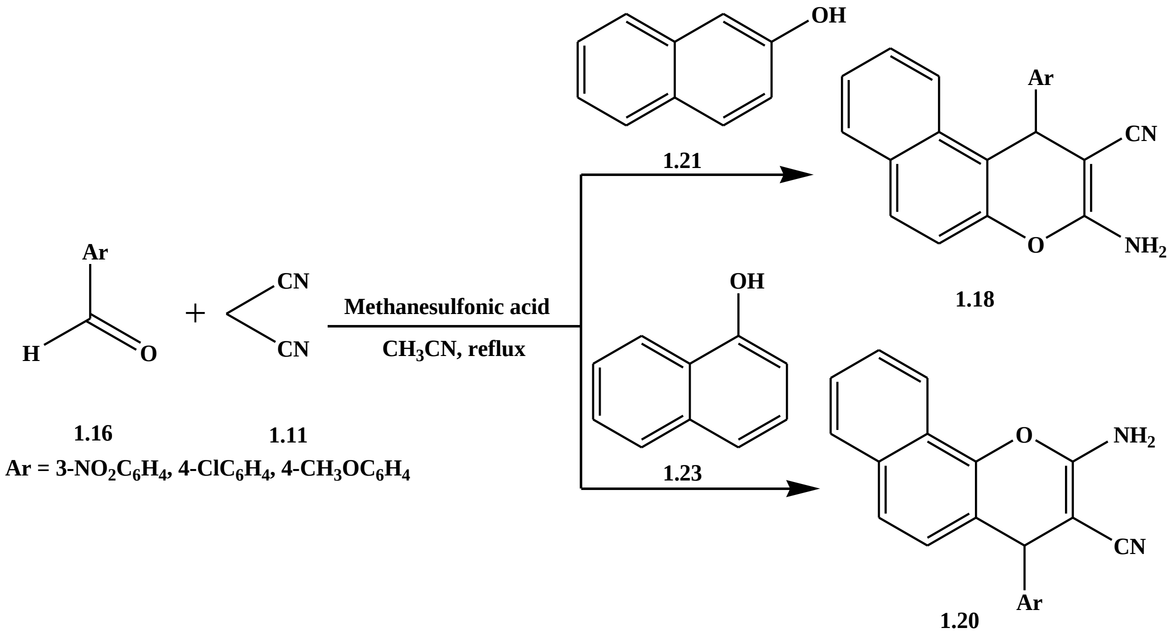
Scheme 1.6: Synthesis of 2-amino-4*H*-chromenes under (MW) in aqueous medium.

In 2007, new 2-amino-4*H*-chromene derivatives were synthesized by the reaction of malononitrile **1.11** or ethyl cyanoacetate **1.29** with salicylaldehyde **1.30** in the presence of a catalytic amount of amberlyst A-21 in ethanol as a solvent at room temperature to afford the **1.31** and **1.32** compounds in high yield **(Scheme 1.7)**.



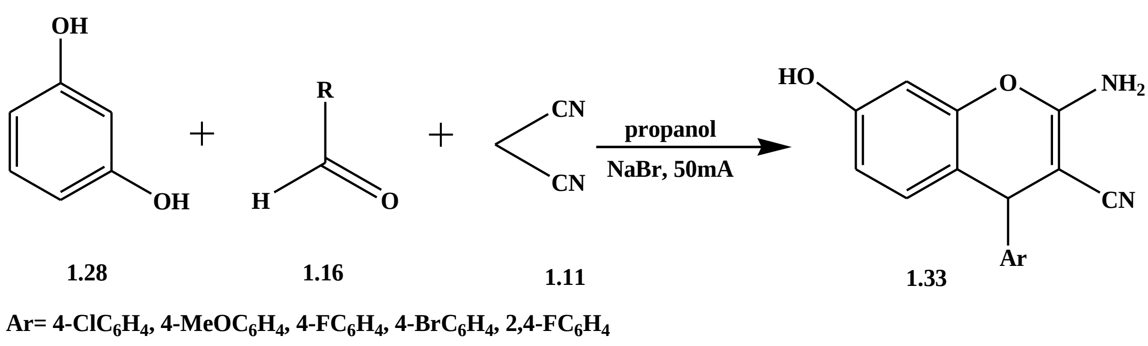
Scheme 1.7: Synthesis of 2-amino-4*H*-chromenes using Amberlyst A-21in ethanol.[33]

In 2008, novel of 2-amino-4*H*-chromene derivatives were prepared via combination of malononitrile **1.11**, aromatic aldehyde **1.16** and 1- and 2-naphthol (**1.23**, **1.21**) in the presence of methanesulfonic acid as a catalyst in acetonitrile to given excellent yield of **1.18** and **1.20** compounds **(Scheme 1.8)**.



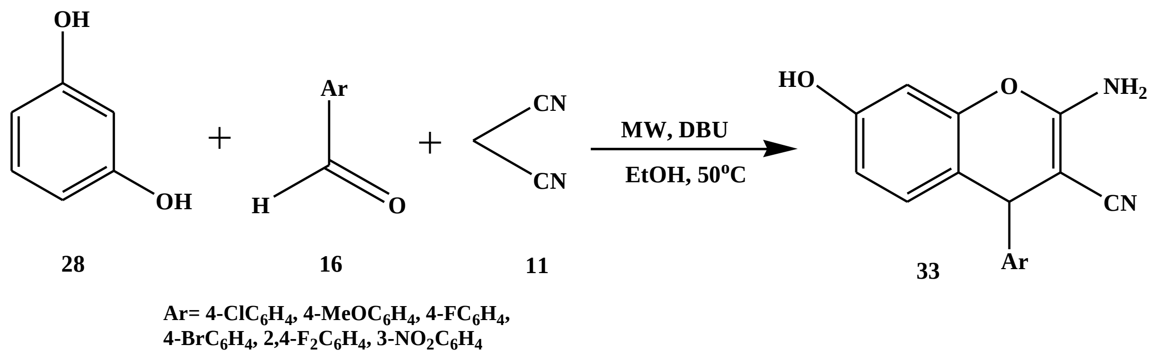
Scheme 1.8: Synthesis of 2-amino-4*H*-benzo[e]chromenes in methane sulfonic acid.[34]

In 2008, novel of 2-amino-4*H*-chromene derivatives were synthesized in good yield by simple electrocatalytic system. The synthetic pathway included condensation between resorcinol **1.28**, malononitrile **1.11** and aromatic aldehyde **1.16** in the presence of dry propanol and current density of 10 mA/cm^2 (I=50 Ma, electrode surface= 5cm^2) to give **1.33** compounds **(Scheme 1.9)**.



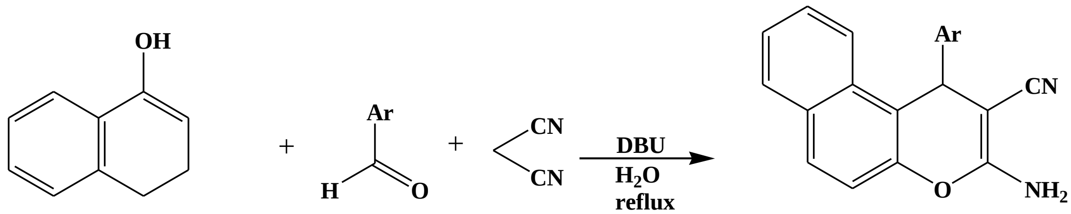
Scheme 1.9: Synthesis of 2-amino-4*H*-chromenes by electrocatalytic system.[35]

In 2010, the 2-amino-4*H*-chromene derivatives **1.33** were prepared by using microwave (MW) by condensation between resorcinol **1.28**, malononitrile **1.11** and suitable aromatic aldehyde **1.16** in ethanolic solution. The authors used 1,8-diazabicyclo (5.4.0) undec-7-ene (DBU) as catalyst for this reaction **(Scheme 1.10)**.



Scheme 1.10: Synthesis of 2-amino-4*H*-chromenes by using DBU under MW.[36]

Khurana *et al.* reported the synthesis of 2-amino-4*H*-benzo[*h*]chromene derivatives **1.34** in 2010. The synthetic pathway included condensation between malononitrile **1.11**, aromatic aldehydes **1.16**, and 1-naphthol **1.23** in the presence of 1, 8-diazabicyclo (5.4.0) undec-7-ene (DBU) as a catalyst. This method was useful due to the short reaction time and mild conditions **(Scheme1.11)**.

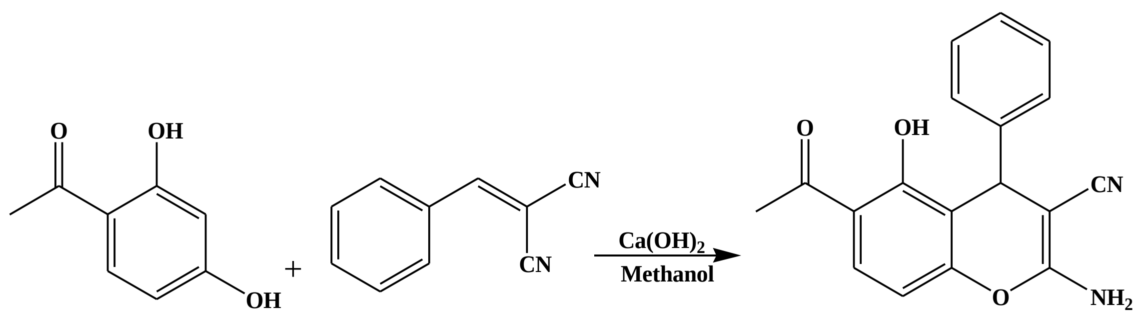


1.23 1.16 1.11 1.34

Ar = 4-BrC_6H_4, -4-$CH_3C_6H_4$, 3,4,5-$(CH_3)_3C_6H_2$, 3,4-$(CH_3)_2C_6H_3$

Scheme 1.11: Synthesis of substitutes 2-amino-4*H*-chromenes by DBU in water.[37]

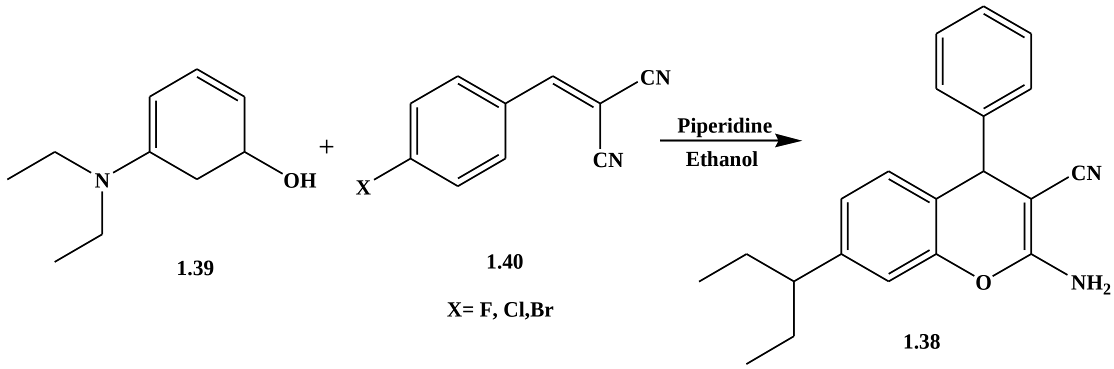
In 2011, 2-amino-5-hydroxy-4*H*-chromene **1.35** was prepared by one pot synthesis of 1-(2, 4-dihydroxyphenyl) ethanone **1.36** and 2-benzylidene malononitrile **1.37** in the presence of calcium hydroxide in methanol at room temperature **(Scheme 1.12)**.



1.36 1.37 1.35

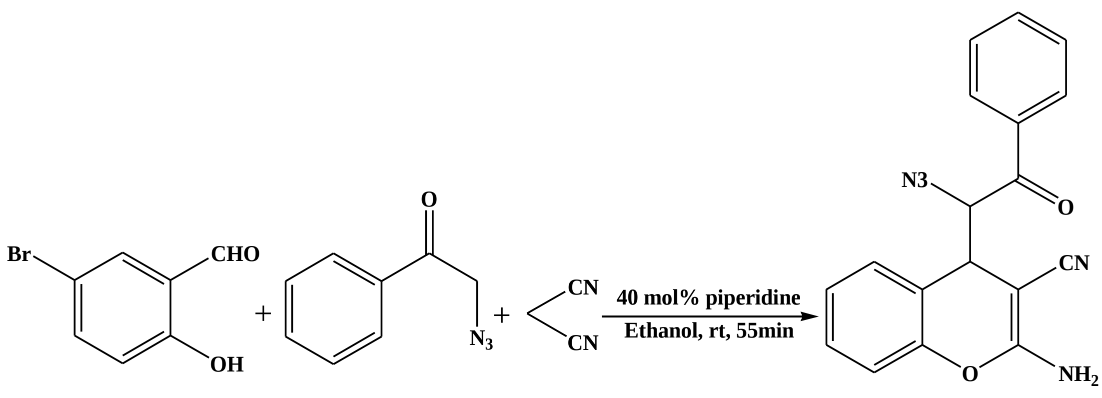
Scheme 1.12: Synthesis of 2-amino-4*H*-chromene in the presence of calcium hydroxide.[38]

In 2011, 2-Amino-4-(4-halophenyl)-7-(diethylamino)-4*H*-chromene-3-carbonitrile **1.38** compounds were prepared by reaction between 3- *N*, *N*-diethylaminophenol **1.39** and ethyl α-cyanocinnamonitriles **1.40** in ethanol and used piperidine as catalyst **(Scheme 1.13)**. Some of these derivatives have cytotoxic effect and antimicrobial activity.[39]



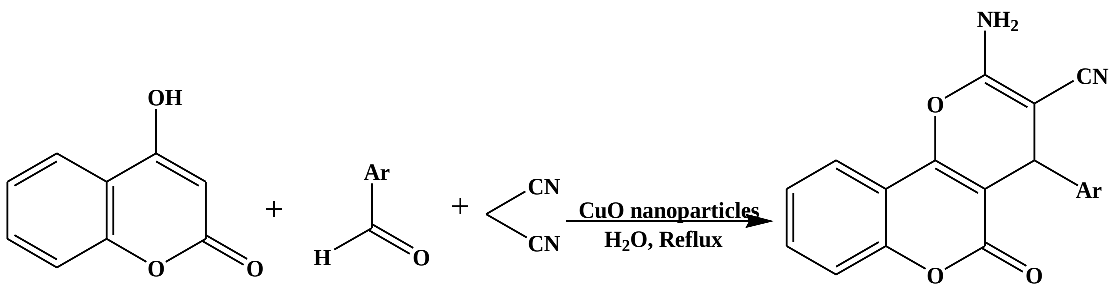
Scheme 1.13: Synthesis of 2-amino-4*H*-chromene in the presence of piperidine.

Babu *et al* reported the synthesis of azido 2-amino-4*H*-chromene **1.41** in 2011. The synthetic pathway included condensation between 3-bromesalicylaldehyde **1.42**, malononitrile **1.11** and phenyl azide **1.43** in ethanolic piperidine solution at room temperature **(Scheme 1.14)**.



Scheme 1.14: Synthesis of azido 2-amino-4*H*-chromene in the presence of piperidine.[40]

In 2011, another one-pot synthetic route has been carried out by reaction of 4-hydroxycoumarin **1.15**, aromatic aldehyde **1.16** and malononitrile **1.11** in water to afford the 3,4-dihydropyrano chromenes **1.17** in high yield using CuO nanoparticles as a catalyst **(scheme 1.15)**

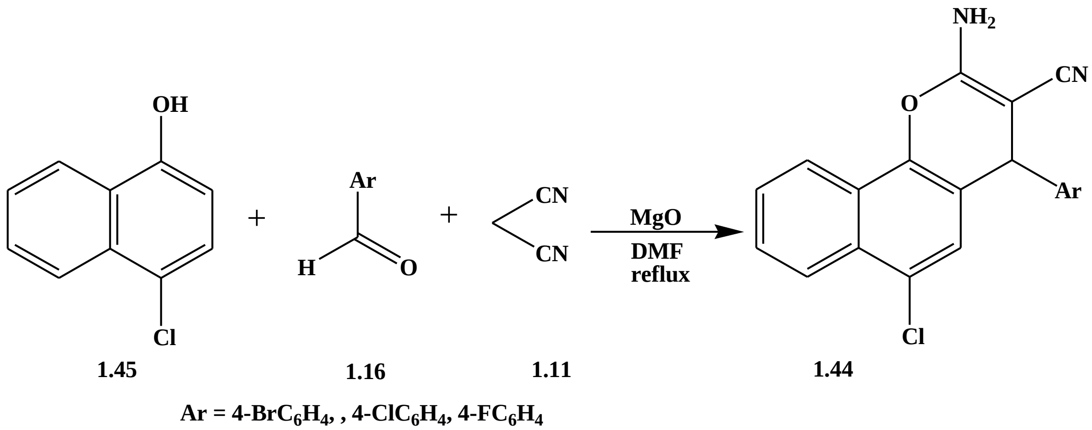


1.15 **1.16** **1.11** **1.17**

Ar = C_6H_5, 4-$NO_2C_6H_4$, 3-ClC_6H_4, 2-$CH_3C_6H_4$, 4-$CH_3OC_6H_4$

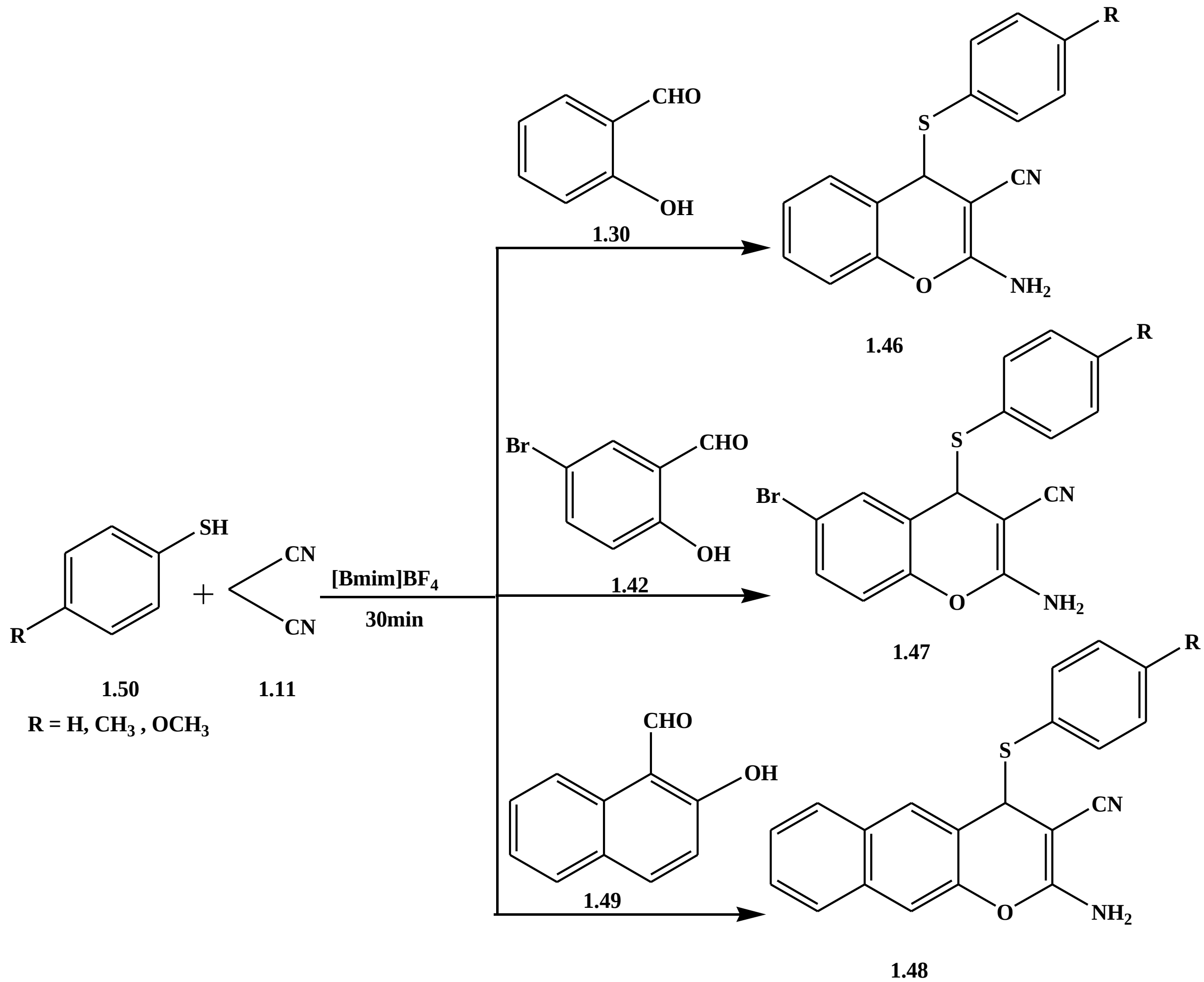
Scheme 1.15: Synthesis of 2-amino-3-cyano-4*H*-chromenes using CuO nanoparticales.[41]

In 2011, Sheibani *et al*. reported the synthesis of 2-amino-3-cyano-4-aryl-4*H*-benzo[*h*]-chromenes **1.44** by condensation between malononitrile **1.11**, 4-chloro1-naphthol **1.45** and substituted aromatic aldehydes **1.16** under reflux and catalyzed by MgO in DMF **(Scheme 1.16)**. Some of these synthesized compounds were evaluated for their anxiolytic activities.[42]



Scheme 1.16: One-pot synthesis of substitutes 2-amino-3-cyano-4-aryl-4*H*-benzo[*h*] chromenes in MgO.

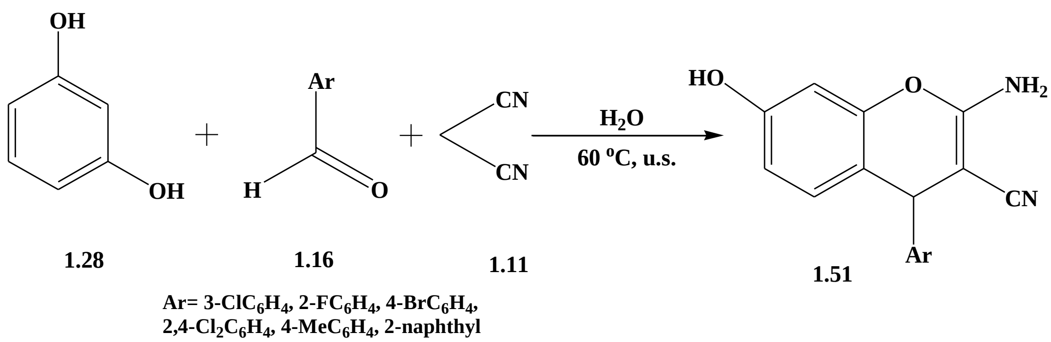
In 2012, 2-amino-3-cyano-4- arylsulfanyl-4*H*-chromenes (**1.46, 1.47, 1.48**) were synthesized by condensation between malononitrile **1.11**, substituted salicylaldehydes (2-hydroxynaphthalene-1-carbaldehyde **1.49** or 5-bromo-2-hydroxybenzaldehyde **1.42** or 2-hydroxybenzaldehyde **30**) and thiols **1.50** in the presence of [Bmim]BF_4 at room temperature **(scheme 1.17)**.



Scheme 1.17: Ionic liquid-catalyzed multicomponent synthesis of 2-amino-3-cyano-4-arylsulfanyl-4*H*-chromenes.[43]

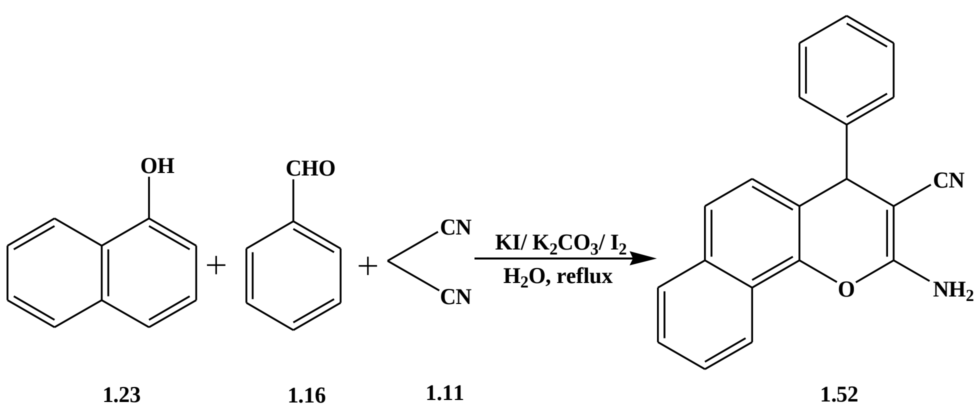
In 2013, novel 2-amino-7-hydroxy-4*H*-chromenes **1.51** were prepared by multi component reaction between resorcinol **1.28**, malononitrile **1.11**, and substituted aryl aldehyde **1.16** without a catalyst in water medium using ultrasound irradiation **(Scheme 1.18)**.

This methodology displayed several advantages over other procedures. It is green, under mild conditions, fast reaction time, and higher yield and selectivity without need for a transition metal or base catalyst.[44]



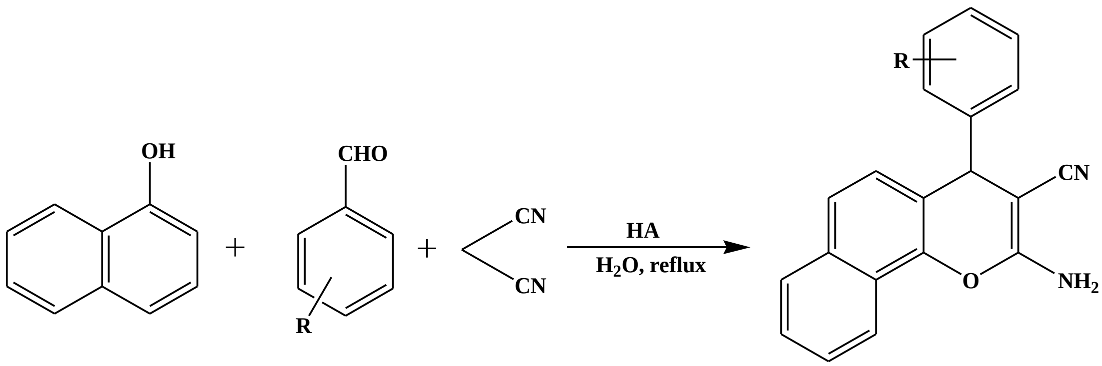
Scheme 1.18: Synthesis of 2-amino-7-hydroxy-4*H*-chromenes under ultrasonic in water.

Sinha *et al.* prepared 2-amino-3-cyano-4-phenyl-4*H*-benzo[*h*]chromene **1.52** in 2013, by condensation between malononitrile **1.11**, benzaldehyde **1.16** and 1-naphthol **1.23** in water to produce the desired products **(Scheme 1.19)**.



Scheme 1.19: Synthesis of 2-amino-4-phenyl-4*H*-benzo[*h*] chromene-3-carbonitrile.[45]

Several of novel 4*H*-benzo[*h*]chromene derivatives **1.20** were synthesized in 2013, by condensation between malononitrile **1.11**, substituted aryl aldehydes **1.16** and 1-naphthol **1.23** in water medium under reflux and catalyzing by hydroxyapatite (HA) or sodium modified hydroxyapatite to afford good yields of products **(Scheme 1.20)**.

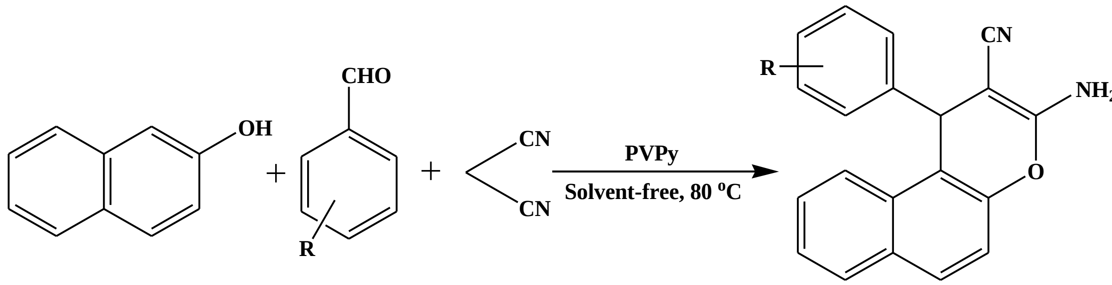


1.23 **1.16** **1.11** **1.20**

R = -Cl, -NO$_2$, -CH$_3$, -OCH$_3$

Scheme 1.20: Hydroxyapatite-catalyzed one-pot synthesis of 4*H*-benzo[*h*]chromenes.[46]

In 2013, several new 2-amino-3-cyano-4*H*-chromene derivatives **1.18** were prepared by condensation of aromatic aldehyde **1.16**, malononitrile **1.11** and 2-aphhol **1.21** in the presence of poly (4-vinylpyridine) catalyst **(Scheme 1.21)**. The main features of the present catalyst are that it is green, commercially available and efficiently recyclable.[47]

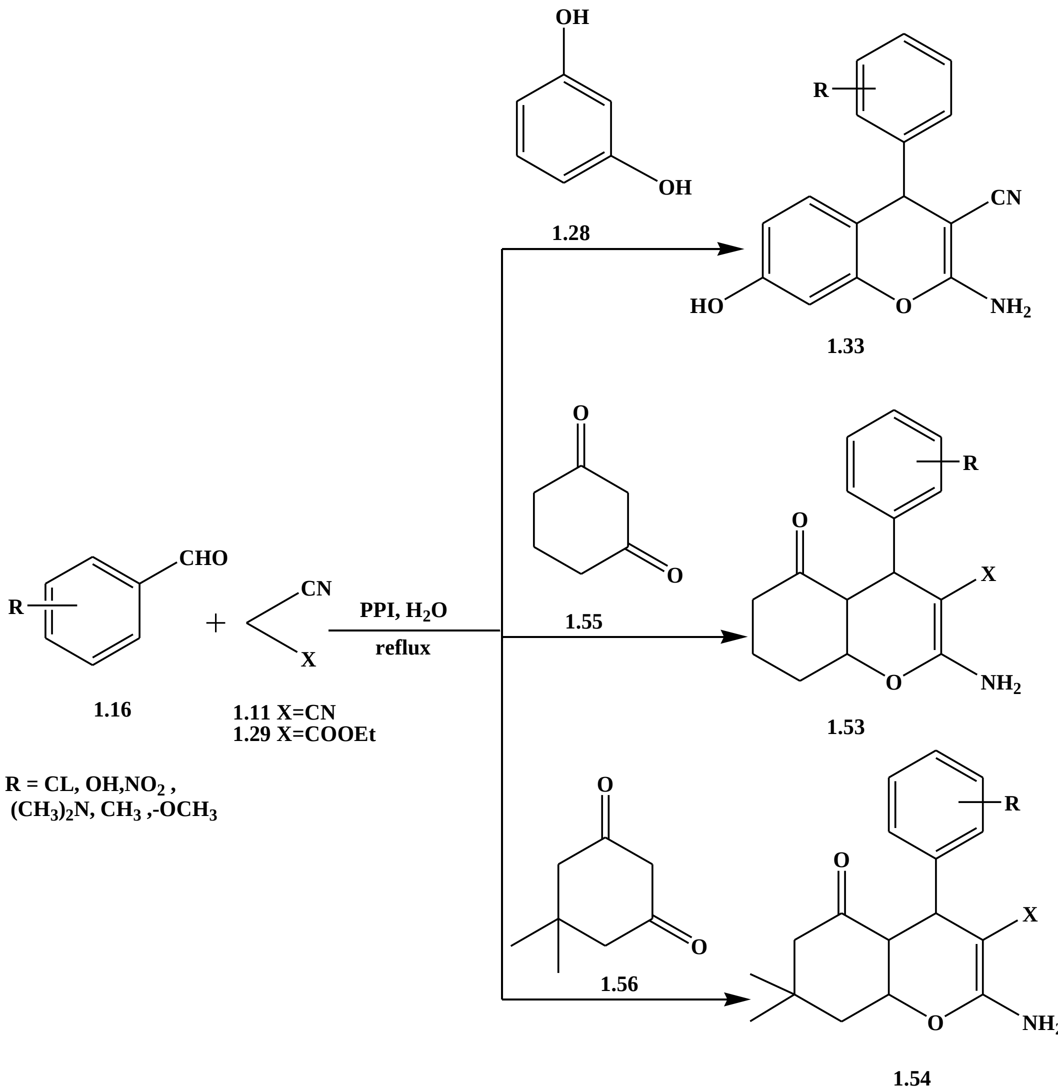


1.21 **1.16** **1.11** **1.18**

R = -Cl, -Br, -NO_2, -CH_3, -OCH_3

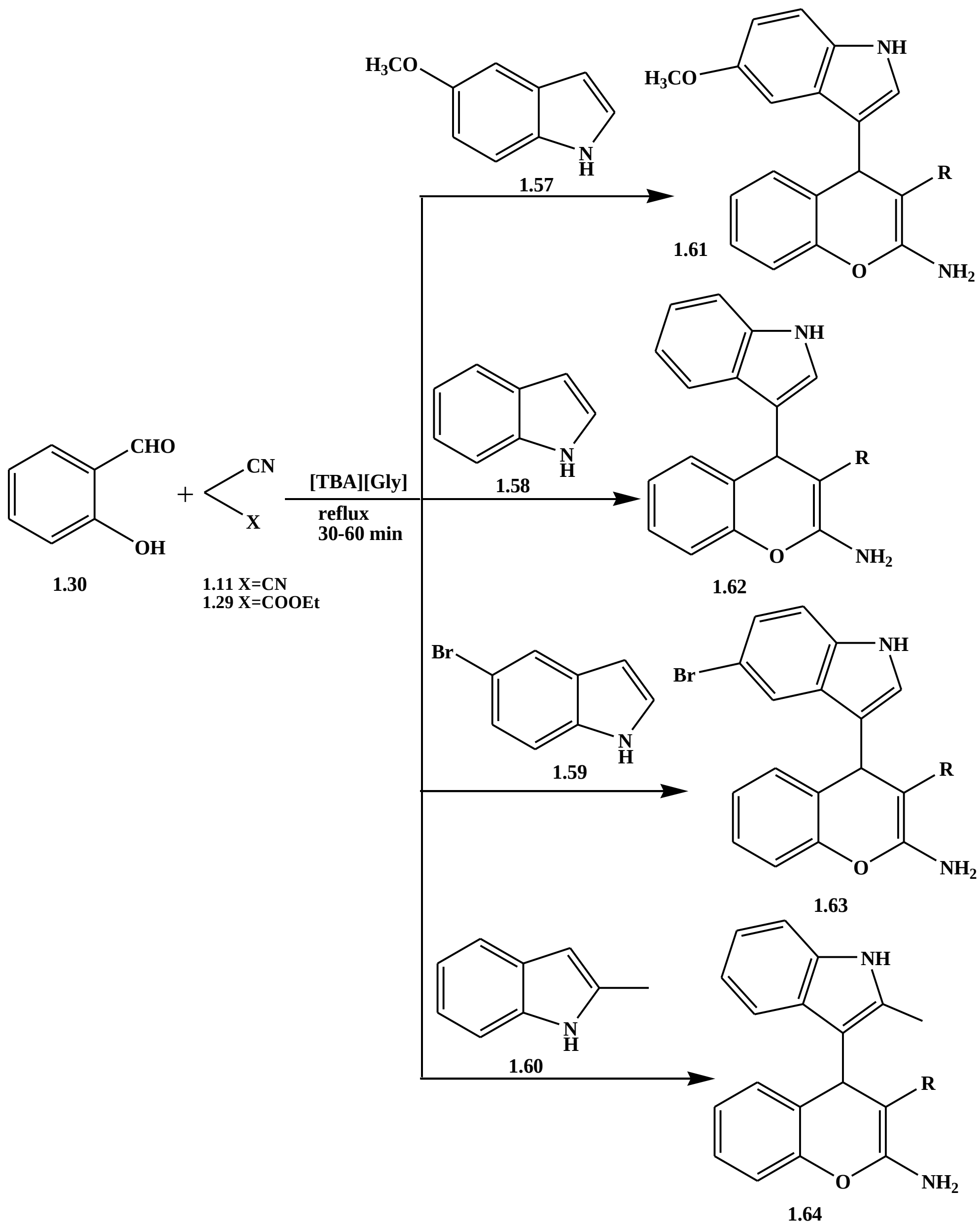
Scheme 1.21: Synthesis of 4*H*-benzo[*h*]chromenes catalyzed by Poly(4-vinylpyridine).

Kiyani *et al*. synthesized 2-amino-4-aryl-7-hydroxy-4*H*-chromene-3-carbonitrailes **1.33** and 2-amino-5-oxo-4-aryl-5,6,7,8-tetrahydro-4*H*-chromenes **1.53, 1.54** by Knoevenagel condensation between suitable aromatic aldehydes **1.16**, malononitrile **1.11** or ethyl cyanoacetate **1.29** with resorcinol **1.28** to afford **1.33** and with cyclohexane-1,3-dione **55** or 5,5-dimethylcyclohexane-1,3-dione **1.56** to give compounds **1.53, 1.54.** In this reaction potassium phthalimide was used as the organocatalyst **(Scheme 1.22)**. The main advantages of the present procedure are that it is green, the short reaction times, high yields and avoiding the use of hazardous organic solvents.[48]



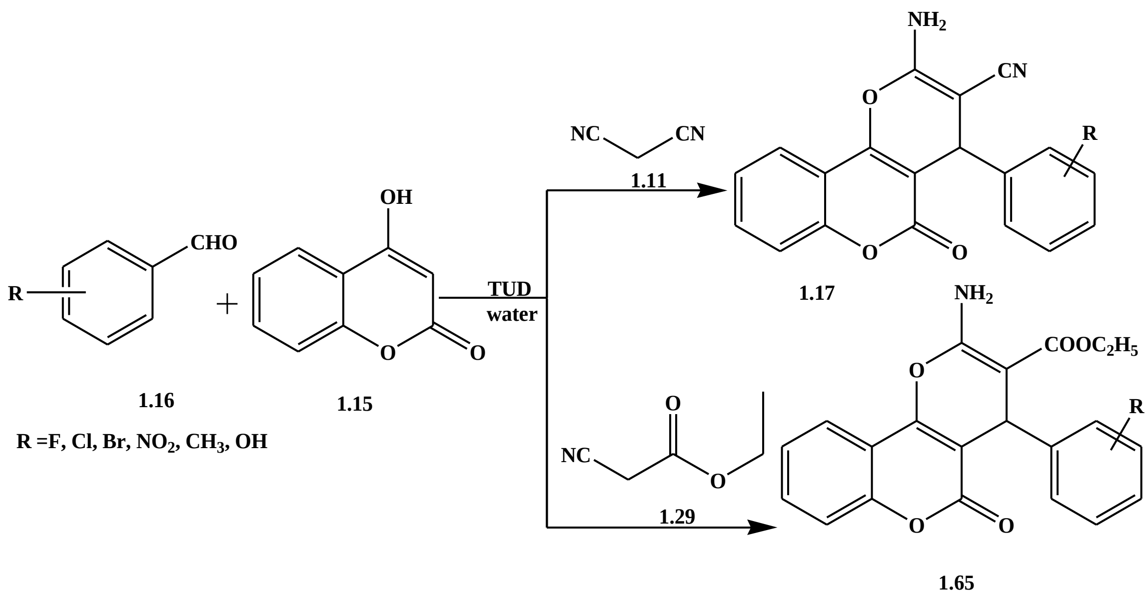
Scheme 1.22: Synthesis of 2-amino-4-aryl-4*H*-chromenes catalyzed by PPI

In 2015, several new of indol-3-yl-4*H*-chromenes were prepared via multi-component reaction between salicylaldehyde **1.30**, malononitrile **1.11** or ethyl cyanoacetate **1.29** and indoles (**1.57**, **1.58**, **1.59**, **1.60**) in the presence of tetra-butyl ammonium glycinate [TBA][Gly] as a catalyst under solvent free conditions at 60 ^{o}C **(Scheme 1.23)** to afford new 2-amino-4*H*-chromene derivatives (**1.61**, **1.62**, **1.63**,**1.64**). This method is metal free and produced high yield The molecular modeling results showed binding interaction of some new synthesized compounds in the active site of Gyrase B in short reaction times.[49]



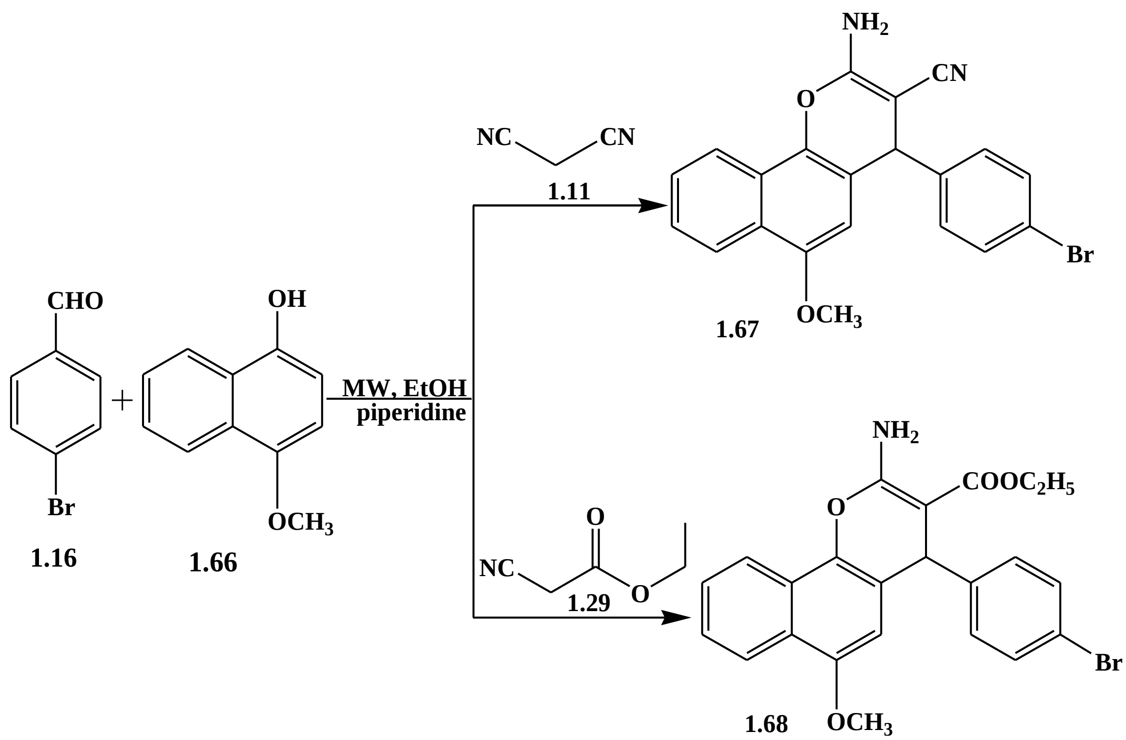
Scheme 1.23: One-pot synthesis of indol-3-yl-4*H*-chromenes catalyzed by [TBA][Gly].

In the same year, Mansoor *et al.* prepared 3,4-dihydropyrano[3,2-*c*] chromene derivatives **1.17**, **1.65** by reaction of substituted aryl aldehydes **1.16**, ethyl cyanoacetate **1.29** or malononitrile **1.11** and 4-hydroxycoumarin **1.15** in the presence of thiourea dioxide (TUD) in aqueous medium at 70 ^{o}C **(Scheme 1.24)**. This technique has several advantages including mild reaction conditions, affording the products in excellent yields and easy purification. In addition, thiourea dioxide is low cost and non-volatilc.[50]



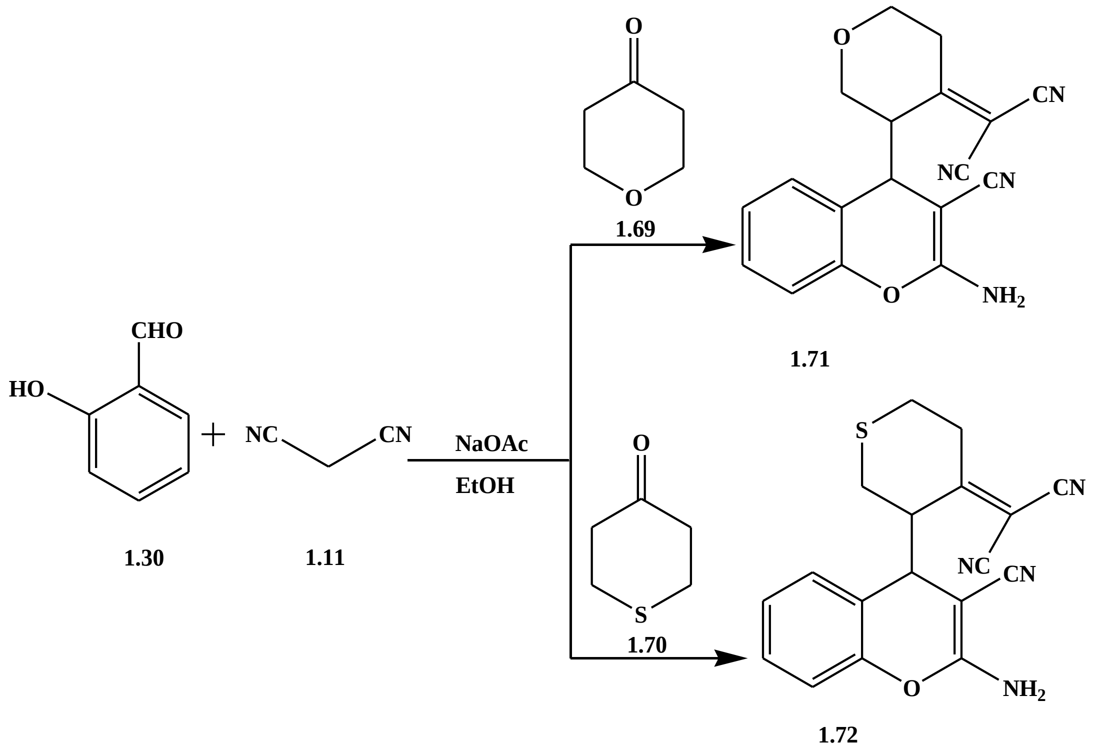
Scheme 1.24: Synthesis of 2-amino-4*H*-chromenes using thiourea dioxide (TUD).

In 2016, novel derivatives of 2-amino-4*H*-benzo[*h*]chromene were synthesized by reacting aromatic benzaldehydes **1.16**, malononitrile **1.11** or ethyl cyanoacetate **1.29** and 4-methoxy-1-naphthol **1.66** in the presence of piperidine in ethanolic solution under (MW) to afford **1.67** and **1.68 (Scheme 1.25)**. Most of synthesized compounds have cytotoxic activity against three cancer cell lines (MCF-7, HCT-116 and HepG2).[51]



Scheme 1.25: Synthesis of 2-amino-4*H*-benzo[*h*]chromenes under (MW) in piperidine.

Elinson *et al.* prepared new 2-amino-4-substituted-4*H*-chromene derivatives in 2016 by pseudo four-component reaction of salicylaldehyde **1.30**, cyclic ketones **1.69** and two molecules of malononitrile **1.11** in the presence of sodium acetate at ambient temperature to afford compounds **1.31** and **1.70 (Scheme 1.26)**.

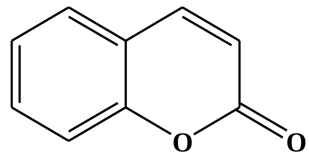


Scheme 1.26: Synthesis of new 2-amino-3-cyano-4*H*-chromenes in the sodium acetate.[52]

1.3 Coumarins

Coumarin (2*H*-1-benzopyran-2-one) **1.73** is classified as a member of the benzopyrone family. It consists of a benzene ring joined to a pyrone ring **(Figure 1.4)**. Coumarins are found in plant products such as vegetables, fruits, seeds, nuts, coffee, tea, and chicory. The first extraction of this naturally occurring compound was published in 1820.[53] Furthermore, coumarins are found in various essential oils such as cinnamon bark oil, cassia leaf oil, and lavender oil.

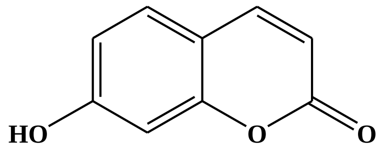
This compound was synthesized and the first synthesis of coumarin was published in 1868.[54, 55]



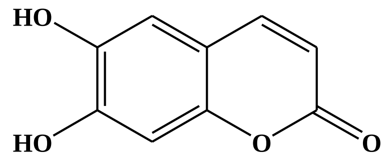
1.73

Figure 1.4: The chemical structures of coumarin

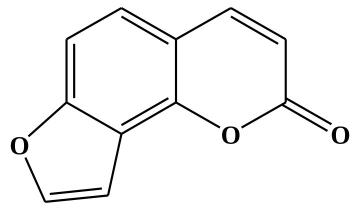
Coumarins are classified into four main subtypes, including simple coumarins, furanocoumarins, pyrancoumarins, and the pyrone-substituted coumarins.[54, 55] Simple coumarins based on the derivatives of the coumarin along with their glycosides, including alkoxylated, hydroxylated, and alkylated. Coumarin, 7-hydroxycoumarin **1.74** and 6,7-dihydroxycoumarin **1.75** are examples of this subtype. Coumarins that consist of a five-membered furan ring attached to the coumarin structure known as furanocoumarins such as angelicin **1.76**. This subtype divided into linear or angular type based on the benzoid position.[54-56] Seselin **1.77** belongs to the pyranocoumarins, which consist of a six membered ring attached to a benzene ring. 4-hydroxycumarin **1.15** attached with pyrone ring that substituted with coumarins are the basic structure to form last coumarin subtype, pyrone substituted coumarins such as warfarin **1.78** as shown in **(Figure 1.5)**.



1.74



1.75



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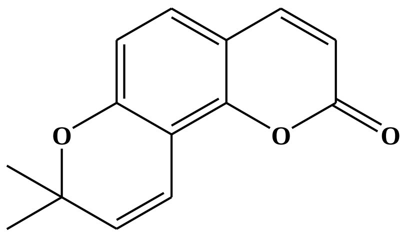

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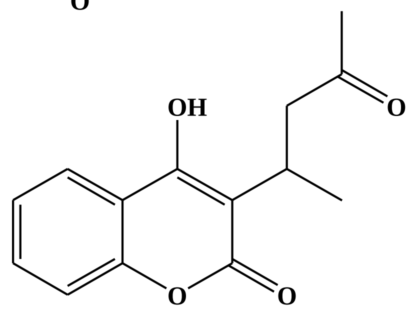
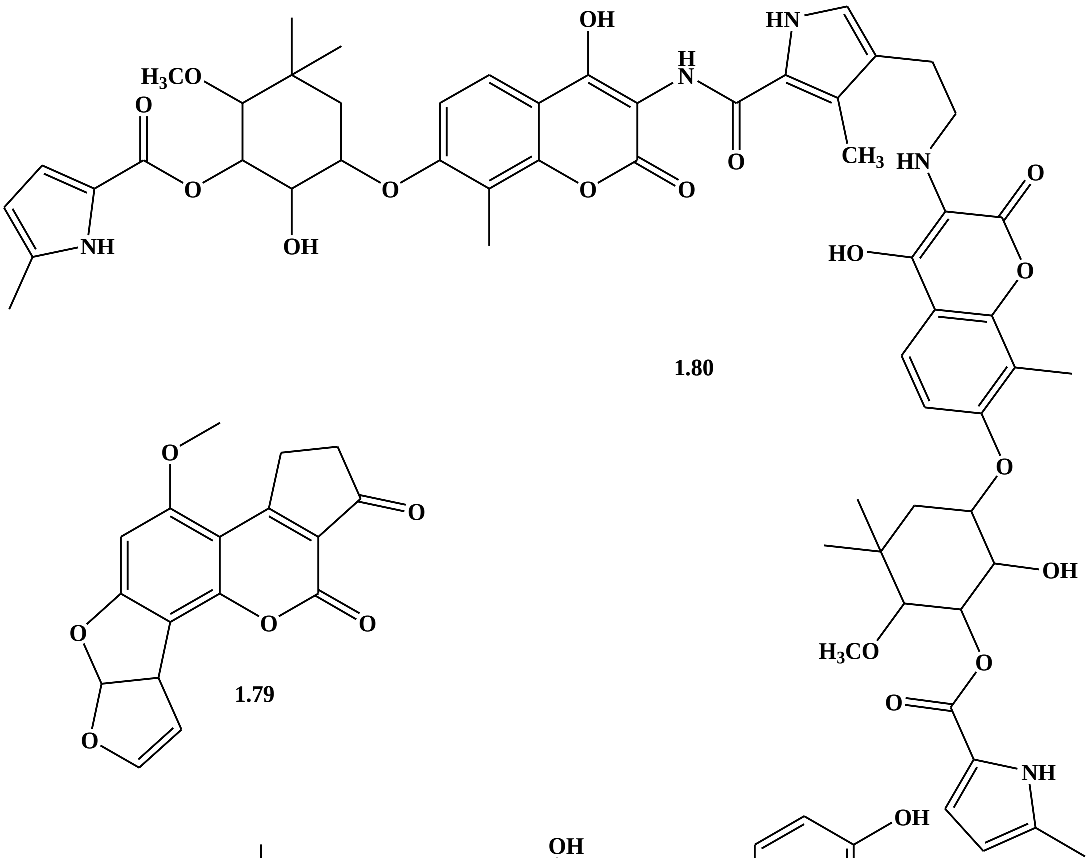


1.78

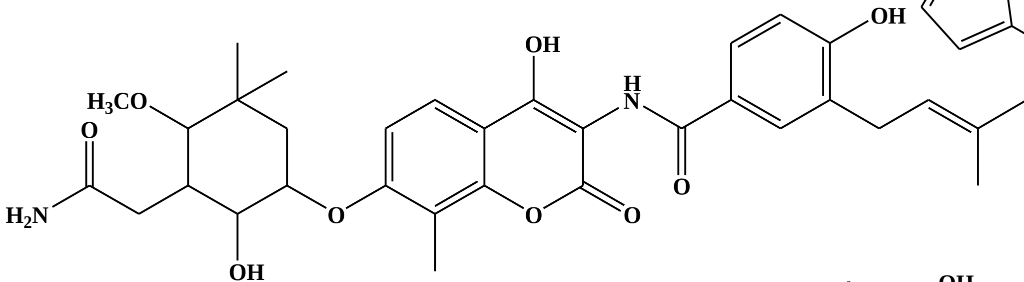
Figure 1.5: The chemical structures of examples of the four main coumarin subtypes.[54-56]

In recent years, coumarins have been classified into six minor types, which have been isolated from the fruits and the stem bark of *Calophyllum dispar* (*Clusiaceae*).[54] Few coumarins have been extracted from microbial sources. Aflatoxin B1 **1.79** is the most commonly occurring member of the aflatoxins group, which possess antifungal activity and are extracted from *Aspergillus*. Furthermore, coumermycin A1 **1.80**, novobiocin **1.81** and clorobiocin **1.82** have been isolated from *Streptomyces* **(Figure 1.6)**. These plant coumarins exhibited antibiotic properties, which are potent inhibitor of DNA gyrase.[54, 55]

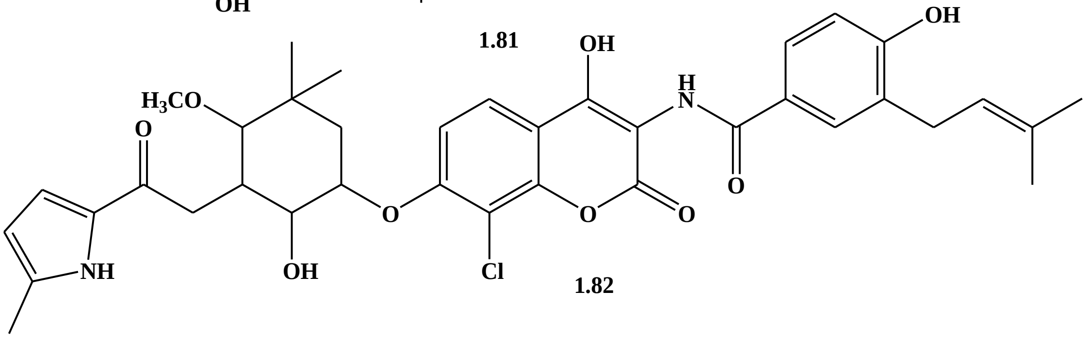


1.80

1.79



1.81



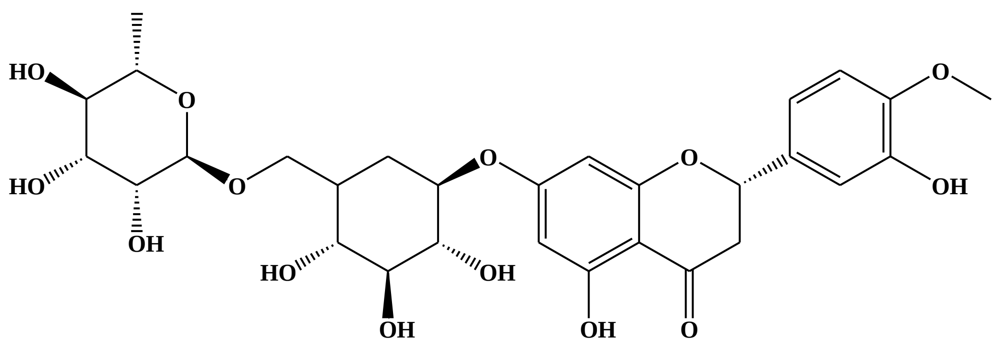
1.82

Figure 1.6: Some important coumarin members isolated from microbial sources.

These natural products have exhibited various biological significance, including anti-inflammatory,[57,58] antifungal,[59] antiviral,[60] anticancer,[61,62] and anti-HIV[63] properties. Furthermore, coumarins have been used as a chemopreventitive agent, mainly against breast cancer in animal models.[64, 65, 66]

1.4 Flavanone

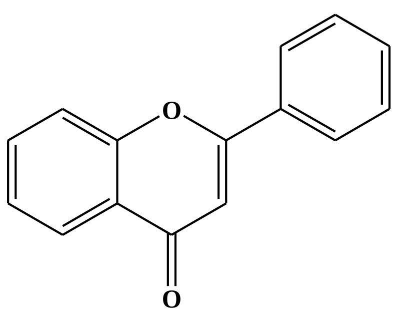
Some natural compounds are very similar in structure and biological activity. These naturally occurring compounds were used for medicinal purposes. Flavonoids are one of these compounds, which are found in plants foods such as fruit, vegetables, tea, and legumes.[67] First experiment described the biological activity of flavonoid was discovered in 1936 by Rusznyak and A. Szent-Gyorgyi. This flavonoid was then named vitamin P **1.83**, which were extracted from *Hungarian red pepper* **(Figure 1.7)**.



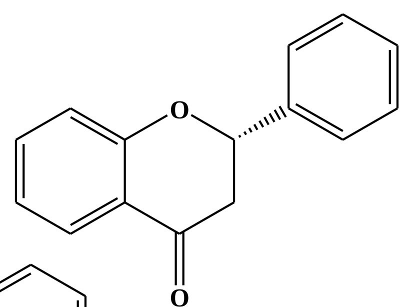
1.83

Figure 1.7: Chemical structure of vitamin P

Five decades later, researcher studied the benefits of flavonoids in the treatment of a various diseases, including pregnancy toxaemia, rheumatic fever, diabetes and cancer. From 1980 to 1990, researchers discovered that flavonoids have variety in biological actions as mutagenic agents, antioxidants and pro-oxidants. A year later, the antioxidant activity of this group was discovered *in vitro*. However, few years later the studies showed that the antioxidant activity of flavonoids *in vivo* could not account for the overall actions attributed to them.[68] Flavonoids have widespread classification, over more than 10,000 structures, and are subdivided into flavone **1.84**, flavanone **1.85**, flavanonol **1.86**, flavan **1.87** and anthocyanidin **1.88 (Figure 1.8)**. Flavanones are considered as a class of flavonoid group, which occur naturally in many fruits and vegetables especially those with dark colors. Flavanones are glycosylated by a disaccharide at position seven to give flavanone glycosides.[68-71]



1.84



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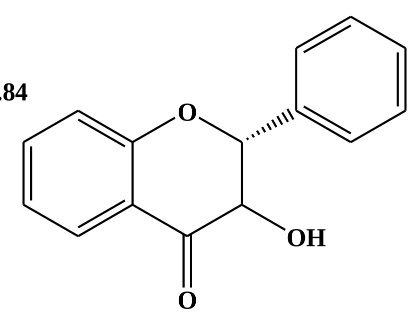
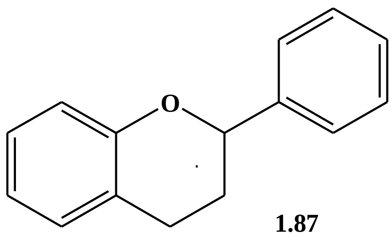


1.86



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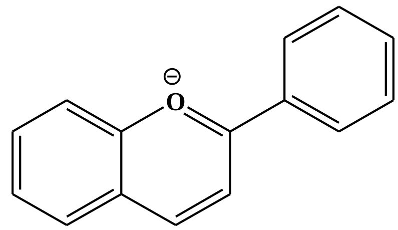
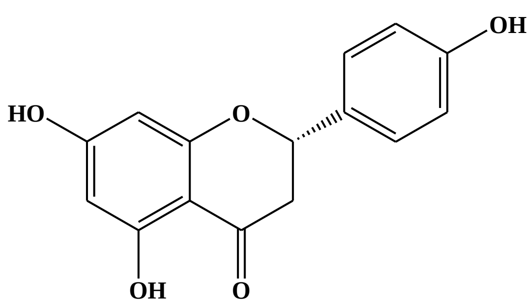


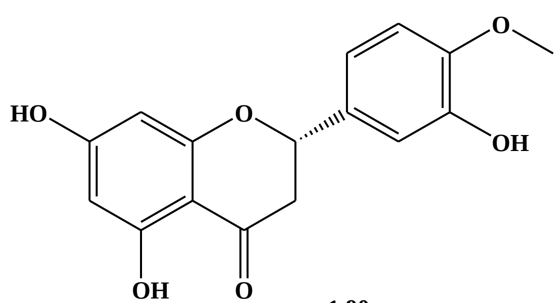
1.88

Figure 1.8: Some chemical structures of flavonoid subtypes.

The main aglycones of flavanones are naringenin **1.89** and hesperetin **1.90 (Figure 1.9)**, but they are more commonly found in glycosides[71]. Most of these compounds have biological significance such as anti-inflammatory, antimicrobial, cytotoxic, and antioxidant.[69-71] However; few studies have been published and described the biological activities of flavanones. Therefore, the purpose of this research was to synthesize a new bioactive compound based on flavanone.



1.89

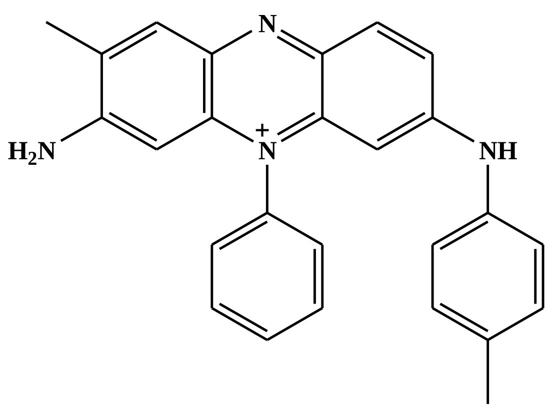


1.90

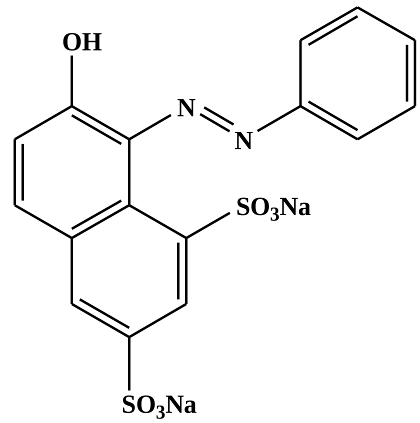
Figure 1.9: Chemical structures of the main aglycones of flavanones.

1.5 Azo chromophores

Over the years, azo dyes have been considered as an important class of organic colorant, which are based into two classes, named pigments and dyes. The key distinction is that pigments are insoluble in the medium while dyes are soluble in the medium to which they are added. Both types have widespread of commercial classifications, and have been used for manufacturing purposes. Azo dyes are compounds that contain at least one conjugated azo group (-N=N-) joined to a methine, aromatic, heterocyclic system by sp^2-hybridization.[72,73] Azo groups are relatively strong and chemically stable.[74] Azo compound structures are composed of two main parts. The first part contains at least one or more auxochrome moieties such as $-NH_2$, -OH, -COOH, $-SO_3H$, while the second part is aromatic groups which are called chromophores. The Auxochrome moieties are responsible for increasing the color intensity and the affinity with the fibers. The dye molecule is often described as a chromogen.[75,76] Azo chromophores exist in two stable isomeric states; the *trans* (E) and the *cis* (Z) forms.[77,78] The azo chromophore can switch between these isomers upon absorption of a photon. Azo dyes were extracted from natural sources based on animals and vegetables by the mid nineteenth century. However, the natural colorants were replaced with manufactured dyes by the beginning of twentieth century. The historical usage of synthetic dyes started in 1856. William Henry Perkin discovered the first commercially synthetic azo dye, named mauveine or aniline purple **1.91**, and orange dye **1.92** was discovered in 1876. **(Figure 1.10)**. [79-81]



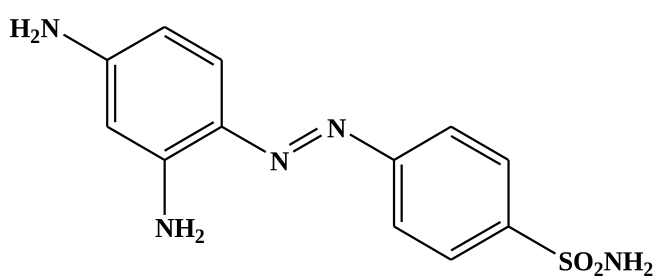
1.91



1.92

Figure 1.10: Chemical structures of some azo compounds isolated from natural sources

Years later, azo dyes have been used for dyeing purposes such as fabric, paper, leather, plastic, cosmetics, among others.[81-83] Furthermore, azo colorants show a variety of biological activity such as antibacterial, antifungal, and anti-HIV activities.[80-85] One of the famous instances of azo dyes is their use in therapeutic treatments of prontosil rubrum **1.93**, which has been widely used in the treatment of serious bacterial illness **(Figure 1.11)**.



1.93

Figure 1.11: Chemical structures of prontosil rubrum.

1.6. Aims of the research

In this thesis, the overall objective is to synthesis and characterize a series of novel chromene derivatives based on three different moieties of biologically active compounds including coumarin, flavanone and azo dyes. After that I will study *in vitro* antimicrobial and anti-cancer for these derivatives. The docking experiments in the active site of DNA gyrase B enzyme of these compounds will be studied. The objective of this study to understand the biologically active of chromenes when combine with other biological active moiety to develop the drugs of bacterial and cancer.

Chapter Two: Synthesis and Characterization of chromene based coumarin derivatives

2.1 Synopsis

A series of novel fluorescent 8-amino-10-phenyl-5-hydroxy-2-oxo-4-propyl-2*H*,10*H*-pyrano[2,3-*f*] chromene-9-carbonitrile derivatives **2.2(a-j)** were synthesized in good yield and with good quantum yields (**Φ_F**). The structures were confirmed on the basis of their spectral data and elemental analysis. Their antimicrobial activity was investigated and tested against seven human pathogen Gram-positive and Gram-negative bacteria: *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* four fungi: *Aspergillus niger, Aspergillus flavus, Fusarium oxisporum, Rhizopus stolonifer* and one mycobacterium, *Mycobacterium tuberculosis*, using the agar well diffusion method and minimum inhibitory concentrations were reported. The fluorescence properties of these compounds afford unique opportunities to examine their intracellular localization. All of the designed compounds showed significant potent antimicrobial activities against most bacterial strains compared to reference drugs. The most active novel compounds **2.2f** and **2.2g** (IZ, around 25 mm and MIC, 0.49-3.9 μg/mL) were subjected to different molecular modeling protocols for study of their activity mechanisms, due to their structural similarity to reference drugs clorobiocin and novobiocin. The docking experiments in the ATP binding pocket of DNA gyrase B enzyme revealed that the compounds mostly have the same binding mode as the reference drugs.

Moreover, the cytotoxic activity was also evaluated against four different human cell lines and exhibited more potency than the reference drug.

2.2 Introduction

Infectious diseases are one of the most responsible factors for a significant proportion of deaths worldwide and, according to the WHO, antimicrobial agents are considered to be "miracle drugs" that are used in the treatment of such diseases.[86] Unfortunately, clinically efficacious antimicrobial agents are becoming less effective due to the resistance caused and gradual appearance of drug-resistant strains among infected communities.[87] Therefore, there is an urgent need for the discovery or optimization of novel antimicrobial agents that are active especially against these resistant strains.[88] Coumarins and dihydropyran derivatives are the most important heterocyclic compounds with diverse and interesting biological activities. Coumarins are a group of biologically active molecules occurring extensively in nature with a wide range of molecular modifications.[89] They exhibit a variety of biological effects including antiviral,[57] anticancer,[58,59] anti-fungal,[60] anti-inflammatory[61,62] and anti-HIV[63] properties. They especially show potent antibacterial effects against Gram-positive species.[90] Plant coumarin antibiotics such as novobiocin and clorobiocin have potential antimicrobial activity by affecting the functioning of DNA gyrase, which is the basis for their broad spectrum antibacterial activity.[91,92] The nature and positions of substituents define coumarin activity, and coumarin substitution in positions 3 and 7 is necessary for antimicrobial activity.[93]

Some natural coumarin-based derivatives have been found to possess anti-microbial effects on methicillin-resistant *Staphylococcus aureus* (MRSA).[94,95] Coumarins with alkoxy and chloro substituents were reported as effective compounds against *Escherichia coli*.[96] Furthermore, coumarins act as intermediates for design of several biologically active molecules such as chromenes, coumarones, fluorocoumarins and bromocoumarins. Chromenes are considered one of the privileged medicinal scaffolds that have attracted a great deal of attention.[97-110] In addition to their biological activity, certain chromene molecules have been used to produce highly effective fluorescent dyes for synthetic fibers, daylight fluorescent pigments, electro photographic and electroluminescent devices.[111,112] Due to interest in the anti-microbial activity of coumarins, 5,7 dihydroxy-4-propyl-coumarin **2.1** was used for the generation of novel derivatives of chromene based coumarins and *in vitro* testing of their antimicrobial and cytotoxic effects.

2.2.1 Study rationale

Natural products bearing a 4-hydroxy coumarin scaffold were shown to possess antimicrobial and cytotoxic activities.[91,92,95] **Figure 1** shows their chemical structures with the similarity analysis and alignment resulting in six-point pharmacophore model that is utilized in our design. They overlap in three points and only two others (hydrophobic and hydrophilic regions) that occur closely at positions 7 and 8 that might affect activity. It was assumed that the hydrophobic region is a key region related to antibacterial and cytotoxic activity and hence has been a motivation to synthesize novel derivatives of different lipophilic substituents. In turn, small molecules were designed and tested for potential antimicrobial and cytotoxic activities based upon the above natural products' structure analyses. Novobiocin, clorobiocin, and mammea-type coumarin are compared to target scaffold. These compounds display pharmacophoric resemblance and key pharmacophoric features are highlighted in red or bold lines.

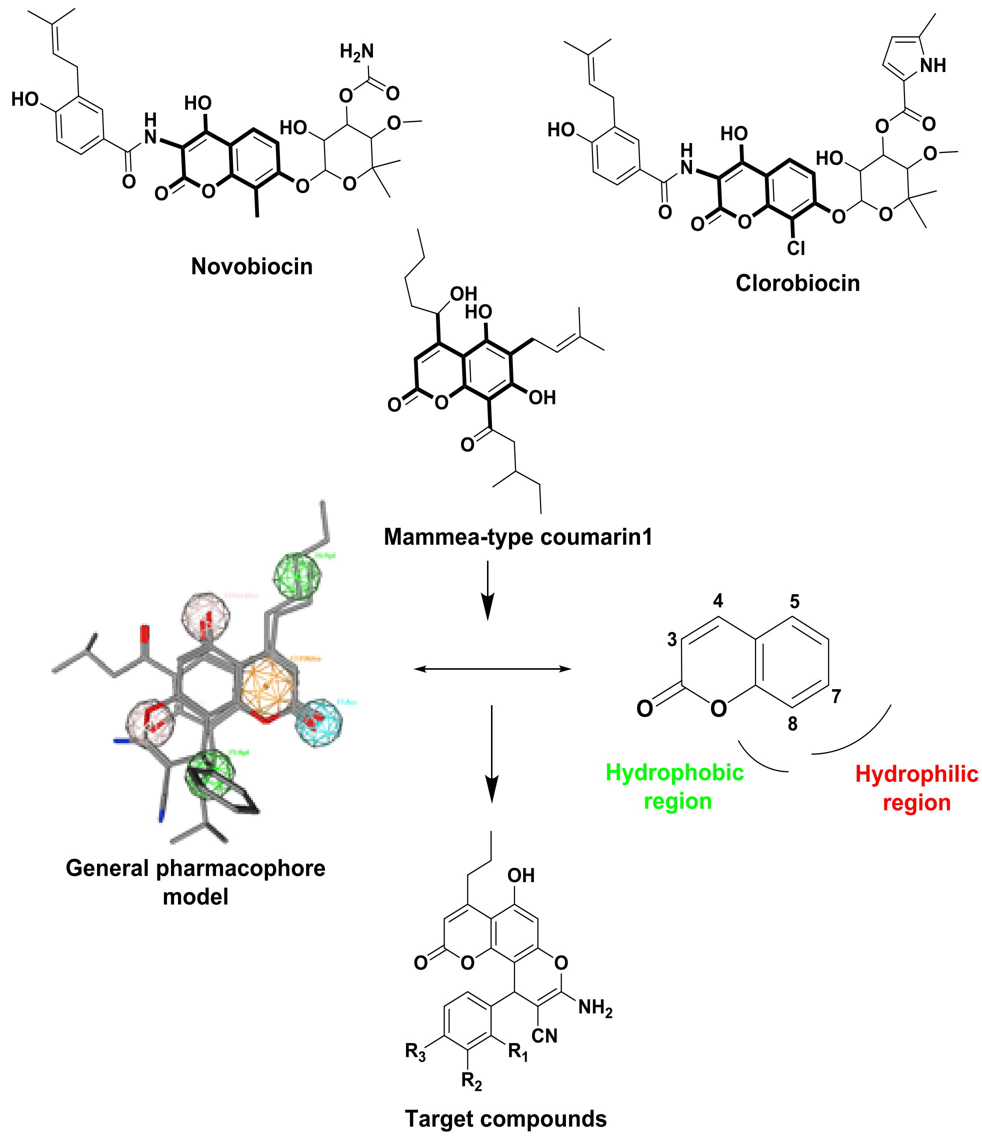
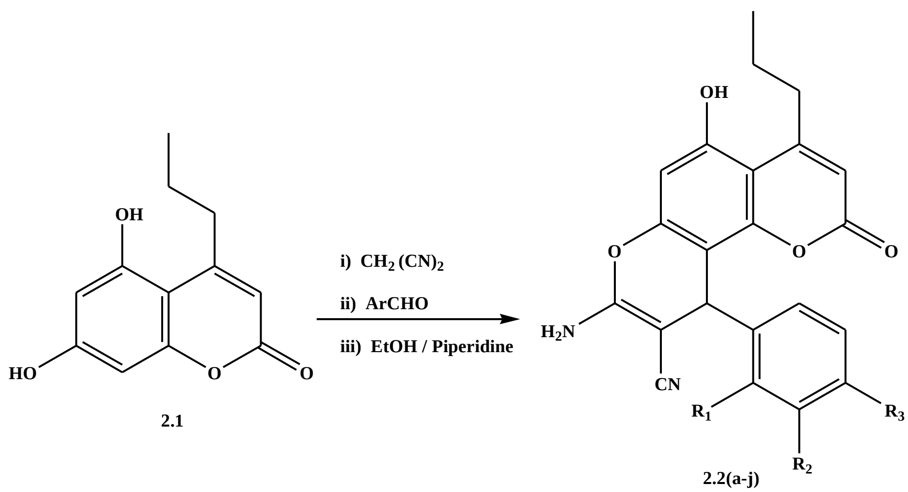


Figure 2.1: Structural similarity of 4-hydroxy coumarin based natural products and target scaffold.

2.3 Results and Discussion

2.3.1 Synthesis and Characterization

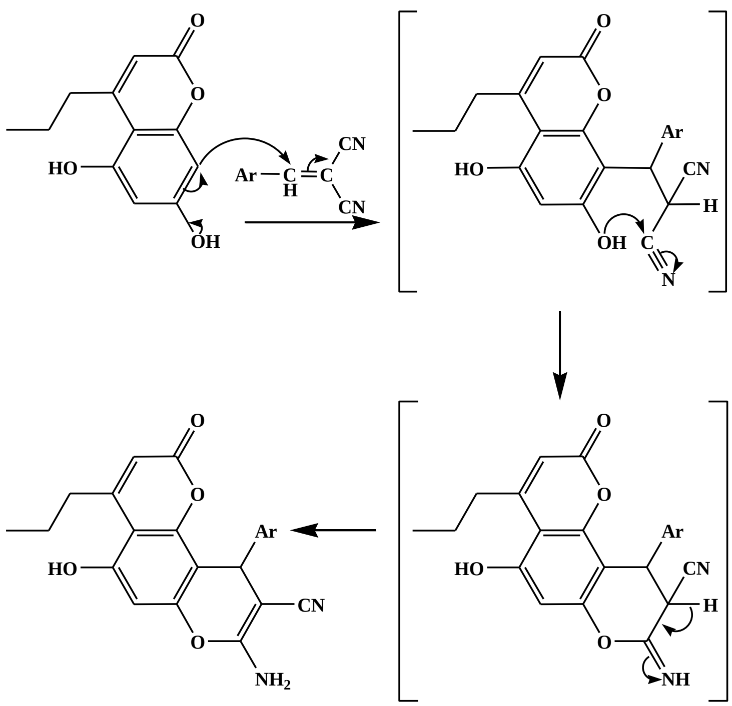
A novel series of 8-amino-10-phenyl-5-hydroxy-2-oxo-4-propyl-2*H*,10*H*-pyrano [2,3-*f*] chromene-9-carbonitrile derivatives **2.2(a-j)** based 5,7-dihydroxy-4-propy-couromin **2.1** were synthesized via three component condensation of 5,7 dihydroxy-4-propyl-coumarin **2.1**, benzaldehyde derivatives and malononitrile in an ethanolic piperidine solution under reflux **(Table 2.1)**. The common method for the synthesis is based on the use of Knoevenagal condensation followed by Michael addition adducts[113], as shown in the following **Schemes 2.1** and **2.2**.



Scheme 2.1: Synthesis of 8-amino-10-phenyl-5-hydroxy-2-oxo-4-propyl-2*H*,10*H*-pyrano [2,3-*f*] chromene-9-carbonitrile derivatives **2.1(a-j).**

Compound No.	R_1	R_2	R_3	Yield%	λ_{abs}	λ_{em}	Φ_F
2.2a	F	H	H	98.29	320-409	448	0.20
2.2b	Cl	H	H	84.23	320-409	450	0.20
2.2c	Br	H	H	96.07	320-414	450	0.26
2.2d	H	Cl	H	79.54	322-415	450	0.40
2.2e	H	Br	H	72.08	322-414	449	0.40
2.2f	H	NO_2	H	94.30	--	--	--
2.2g	H	OH	H	76.47	316-416	448	0.26
2.2h	H	H	n-butyl	88.76	316-416	451	0.31
2.2i	H	H	OH	75.29	316-416	452	0.41
2.2j	H	H	F	80.23	324-417	449	0.54

Table 2.1: The quantum yield (Φ_F), wavelength of emission (λ_{em}) and absorption (λ_{abs}), for synthesized derivatives **2.2(a-j)**



Scheme 2.2: General synthesis and mechanistic pathway of chromene molecules **2.2(a-j)**

The structures of new chromene molecules **2.2(a-j)** were characterized using FT-IR and NMR spectroscopic techniques. For instance, the FT-IR spectroscopy showed characteristic absorption bands between 2181 and 2200 for the CN groups while the NH_2 stretches were in the range of 3398-3409 cm^{-1}. Moreover, the C=O group displayed bands at 1697-1727 cm^{-1} and the OH bands appeared at 3403-3597 cm^{-1}. The ^{1}H NMR spectra of chromene compounds **2.2(a-j)** were obtained in DMSO-d_6. As anticipated, the methyl protons appeared as a triplet set at 0.96-0.98 ppm, while the H10 pyran showed signals at 4.51-5.18 ppm and the amine protons resonated as a singlet at 6.71-7.11 ppm. The singlet at 5.99-6.51 ppm is corresponding to H3, while H6 appeared as singlet between 5.67-6.06 ppm. The rest of the aromatic protons resonate further downfield in the range of 6.63-8.12 ppm. In all spectra, the methylene group directly linked to the coumarin moiety showed two signals as shown in **Figure 2.2** as example of the proton NMR for compound **2.2f**.

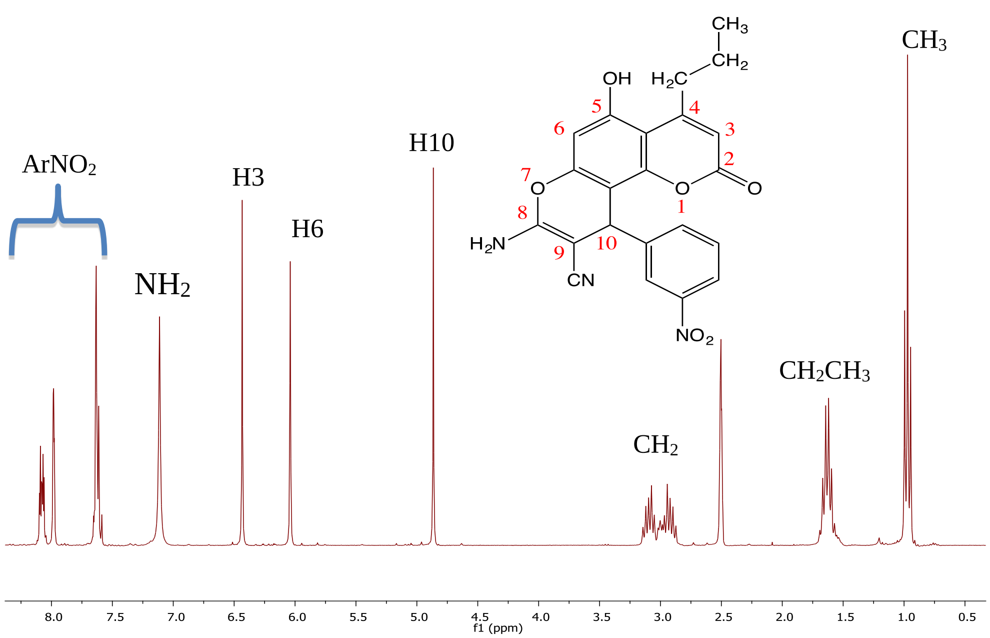


Figure 2.2: ^{1}H NMR of Compound **2.2f**

The ^{13}C NMR spectra of the new molecules showed that the carbon atoms attached to the methyl protons resonate at 14.29-13.28 ppm, while the two methylene carbons resonate at 23.75-22.06 ppm and 38.18-37.25 ppm. The signal at 37.12-36.37 ppm is corresponding to C-4` of the pyran ring, while the quaternary carbon C-2` that is attached to the amine group appeared in the range of 57.78-58.34 ppm. The CN carbon resonated further downfield at 109.84-107.08 ppm and the aromatic CH carbons showed signals between 134.74 and 114.74 ppm as shown in **Figure 2.3**.

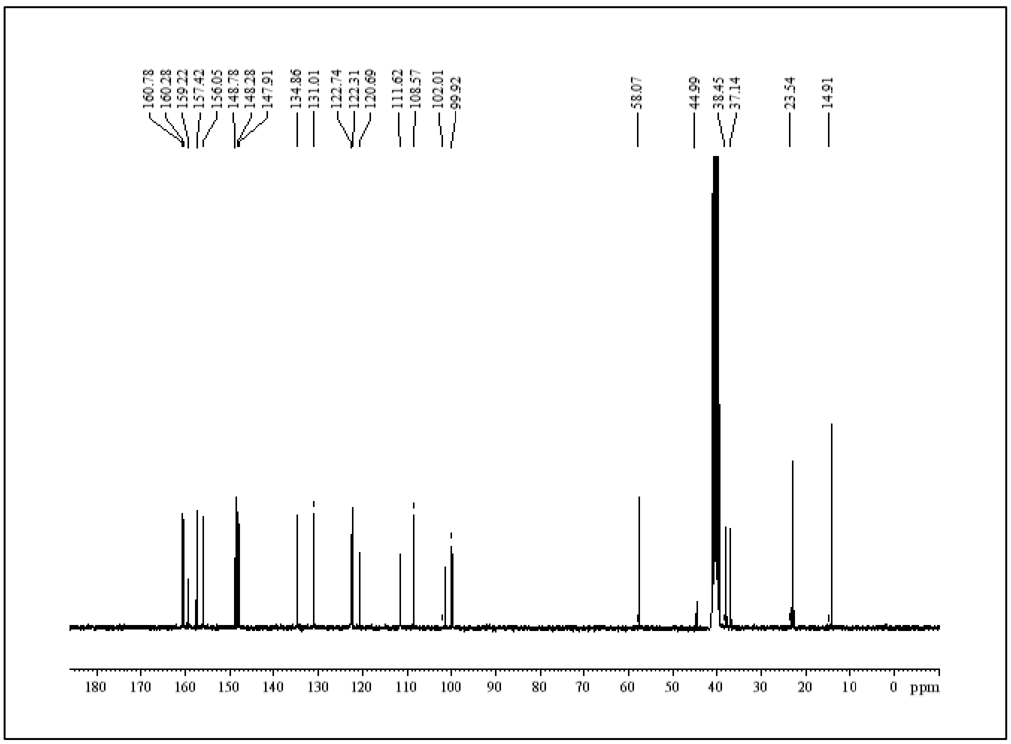


Figure 2.3: ^{13}C NMR spectrum of **2.2f**

The UV-vis spectra of the new chromene compounds **2.2(a-j)** in DMF were found to absorb at two distinct peaks around 322 and 410 nm with almost similar shape as shown in **Figure 2.4**

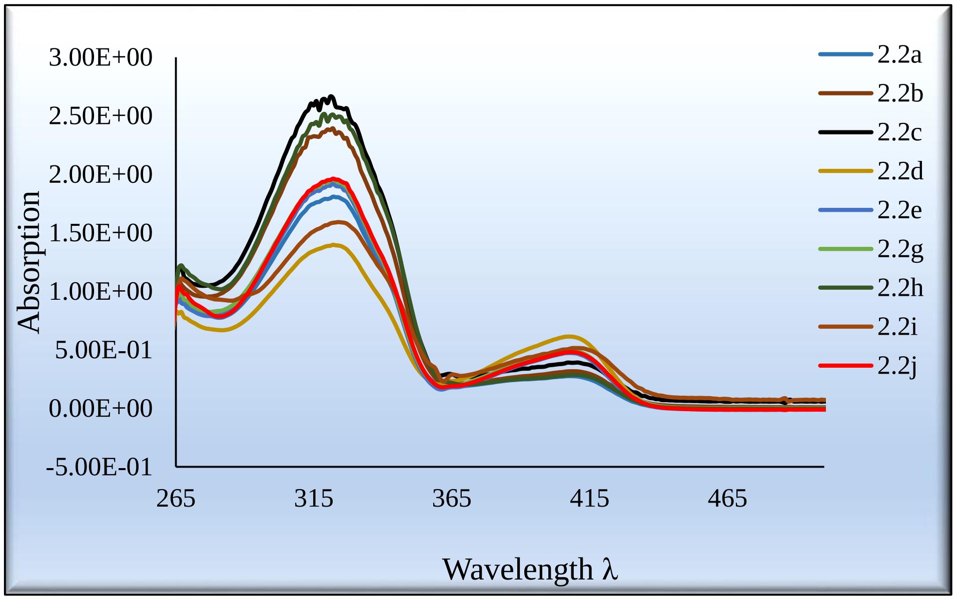


Figure 2.4: UV-vis absorption spectra of compounds **2.2(a-j)** (10µM) in DMF

Also, fluorescence properties of compounds **2.2(a-j)** were examined in DMF solution. Due to their conjugation system, all compounds except **2.2f** exhibited emission wavelengths around 450 nm with excitation λ_{max} at 410 nm, **Figure 2.5**. In contrast, the presence of nitro substituent in **2.2f** quenches the fluorescence of this molecule.[114,115]

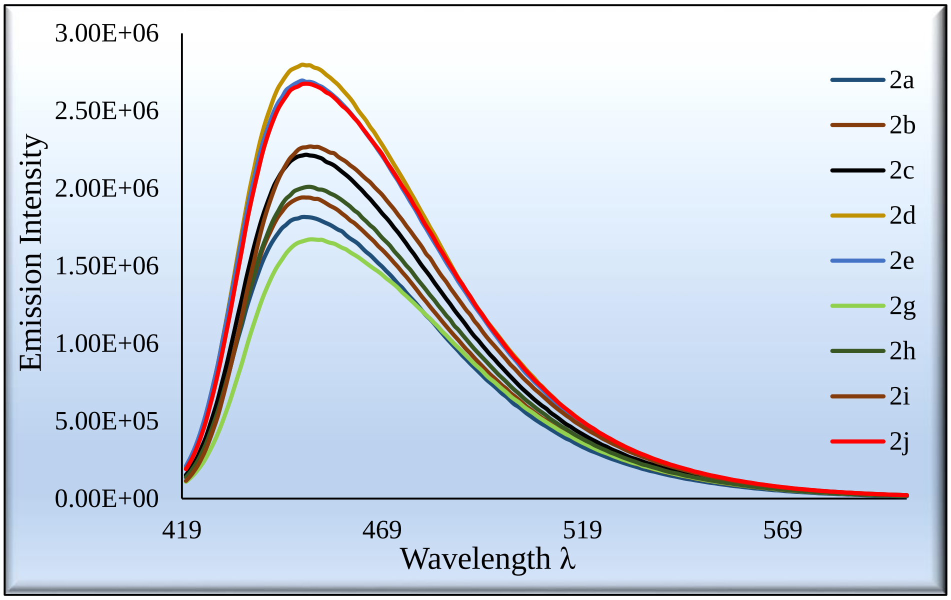


Figure 2.5: The fluorescence spectra of the new chromene compounds **2.2(a-j)** in DMF at a concentration of 10 μM.

The fluorescence spectra of these compounds in different solvents such as dimethyl sulfoxide (DMSO), *N*, *N*-dimethylformamide (DMF), dichloromethane (DCM), acetonitrile (ACN), and acetone (Ace) were consistent with the polarity index (PI) for each solvent.[116] For example, in compound **2.2a** the λ_{max} (emission) in DMSO (PI 7.2) was 452 nm, DMF (PI 6.4) was 450 nm, ACN (PI 5.8) was 442 nm, Ace (PI 5.1) was 441 nm and DCM (PI 3.1) was 430 nm **(Figure 2.6)**.

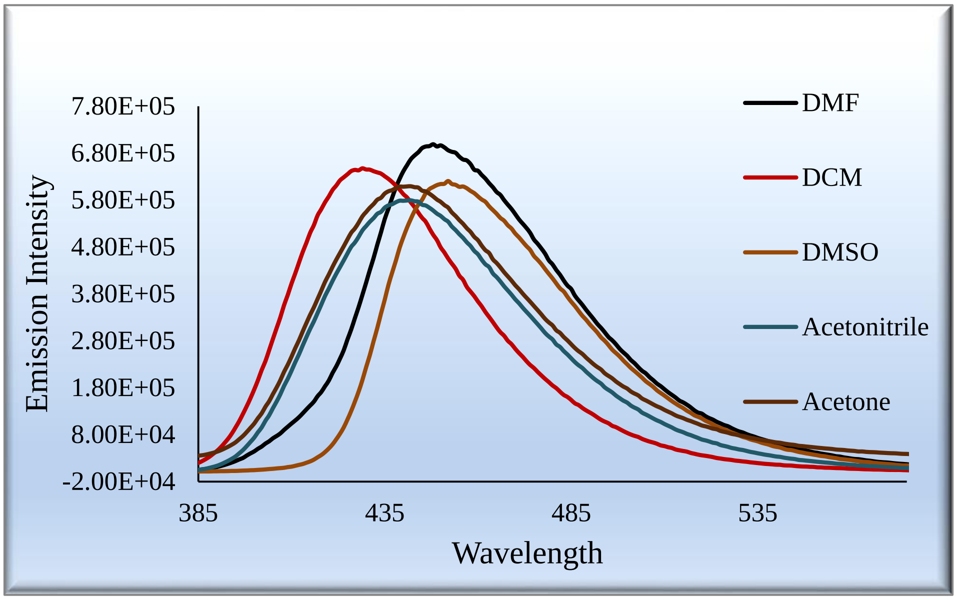


Figure 2.6: The fluorescence spectra of compound **2.2a** in different solvents (10 μM).

2.3.2 Biological screening

2.3.2.1 Antimicrobial Screening

All the newly synthesized compounds **2.2(a-j)** were screened for their antibacterial, antifungal, and antimycobacterium activities via agar diffusion well method.[117] The inhibition zones and minimum inhibitory concentrations (MIC) were determined by serial dilution method.[118] The activity of the synthesized compounds was tested against four Gram-positive bacteria including *Streptococcus pneumoniae* (RCMB 010010), *Bacillus subtilis* (RCMB 010067), *Staphylococcus aureus* (RCMB 000106), and *Methicillin-Resistant Staphylococcus aureus* (MRSA 2658 RCMB), three Gram-negative bacteria including *Pseudomonas aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052), and *Salmonella typhimurium* (RCMB 000106), four fungi including *Aspergillus fumigatus* (RCMB 02568), *Syncephalastrum racemosum* (RCMB 05922*)*, *Geotricum candidum* (RCMB 05097), and *Candida albicans* (RCMB 05036), and finally, against *Mycobacterium tuberculosis* (RCMB 010094-8). Ampicillin, ciprofloxacin, gentamicin, amphotericin B, vancomycin, and isoniazid were used as control drugs.[119,120] The observed inhibition zone (IZ) and minimum inhibitory concentrations (MIC) of the compounds and the reference drugs are given in **Table 2.2, 2.3** and presented in **Figure 2.7, 2.8**. The data showed that most of the tested compounds exhibited appreciable bacterial and fungal inhibition compared to reference drugs. In general, the synthesized compounds were more active against the Gram-negative having IZ values greater than 20. Among the synthesized compounds **2.2(e-j)** were found to be more effective against *E. coli* by IZ range 21 to 24 mm and MIC 0.49 to 3.9 µg/ml compared to reference drugs. Compounds **2.2(e-g)** and **2.2j** were also found to be more active against S.T by IZ 22-26 mm. In the case of antibacterial activity against Gram-positive bacteria, most compounds were found to be comparably active to reference drugs, having a MIC range 3.9-0.49 µg/mL. In particular, **2.2g** exhibited mild inhibitory activity towards MRSA, IZ 21.3 mm and MIC 1.95 µg/mL. The synthesized compounds were moderately or slightly active against the fungal species and *Mycobacterium tuberculosis* (TB). Generally, the investigation of antimicrobial activity of the novel derivatives showed more potency than the reference drugs against Gram-negative bacteria and mild activity towards Gram-positive bacteria, fungi, and mycobacterium.

Table 2.2: Antimicrobial activity of synthetic compounds (IZ, diameter (mm)) (1g/mL).

Compounds	Inhibition Zone Diameter (mm)											
	Gram-positive				Gram-negative			Fungi				TB
	S.P	B.S	S.A	MRSA	P.A	E.C	S.T	A.F	S.R	G.C	C.A	
2.2a	18.2	17.6	16.1	NA	15.6	17.1	17.9	14.2	NA	13.6	11.4	NA
2.2b	19.4	18.1	16.9	NA	16.3	18.2	18.6	16.3	NA	14.7	12.6	NA
2.2c	13.6	16.4	12.4	NA	13.2	15.7	17.3	16.3	NA	18.2	15.4	NA
2.2d	17.3	17.9	13.6	NA	11.2	13.4	16.2	15.4	NA	14.2	13.6	NA
2.2e	22.4	21.3	20.6	18.3	21.3	22.4	22.6	20.1	NA	18.2	17.3	NA
2.2f	24.2	23.6	21.3	19.6	22.4	24.6	25.1	20.3	NA	22.4	19.3	NA
2.2g	20.4	22.6	19.8	21.3	23.4	24.9	26.4	21.3	NA	24.3	20.8	62.5
2.2h	18.7	21.3	20.2	NA	18.3	21.2	21.9	18.6	NA	20.1	18.3	NA
2.2i	21.3	22.1	20.3	NA	18.4	20.3	21.2	20.6	NA	21.2	18.3	NA
2.2j	21.9	21.2	20.1	16.3	20.4	21.6	22.2	19.6	NA	16.9	16.4	NA
Ampicillin	23.8	32.4	26.2	-	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	23.4	26.2	27.4	-	-	-	-	-
Gentamicin	-	-	-	-	17.3	19.9	22.3	-	-	-	-	-
Amphotericin B	-	-	-	-	-	-	-	23.7	19.7	28.7	25.4	-
Vancomycin	-	-	-	20.3	-	-	-	-	-	-	-	-
Isoniazid	-	-	-	-	-	-	-	-	-	-	-	83.2

Mean zone of inhibition in mm from at least three experiments; (triplicate, mean ± SE, with standard errors range 0.01- 0.8). (NA) means no activity. S.P (*Streptococcus pneumoniae*), B.S (*Bacillus subtilis*), P.A (*Pseudomonas aeruginosa*), E.C (*Escherichia coli*), S.T (*Salmonella typhimurium*), A.F *Aspergillus fumigatus* (RCMB 02568), G.C *Geotricum candidum* (RCMB 05097), S.R *Syncephalastrum racemosum* (RCMB 05922), and C.A *Candida albicans* (RCMB 05036) and TB *Mycobacterium tuberculosis* (RCMB 010094-8)

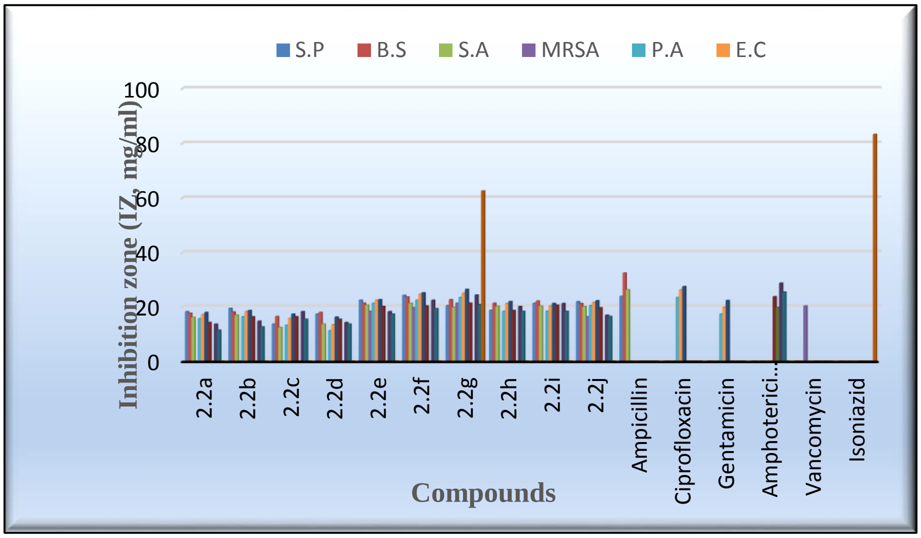


Figure 2.7: Evaluation of Inhibition zone values (IZ) of synthesized derivatives

Compounds	Minimal inhibitory concentration (MIC, µg/ml)										
	Gram-positive				Gram-negative			Fungi			
	S.P	B.S	S.A	MRSA	P.A	E.C	S.T	A.F	S.R	G.C	C.A
2.2e	1.95	1.95	1.95	7.81	1.95	1.95	0.98	3.9	NA	7.81	15.63
2.2f	0.49	0.98	1.95	3.9	1.95	0.98	0.49	3.9	NA	1.95	3.9
2.2g	3.9	1.95	3.9	1.95	0.98	0.49	0.49	1.95	NA	0.49	1.95
2.2j	1.95	1.95	3.9	3.9	3.9	1.95	1.95	3.9	NA	15.63	31.25
Ampicillin	0.98	0.24	0.49	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	0.99	0.59	0.56	-	-	-	-
Gentamicin	-	-	-	-	15.63	3.9	1.95	-	-	-	-
Amphotericin B	-	-	-	-	-	-	-	0.98	3.9	0.49	0.49
Vancomycin	-	-	-	3.9	-	-	-	-	-	-	-

Table 2.3: Antimicrobial activity of synthetic compounds (MIC, µg/ml).

Minimum inhibitory concentration (MIC, mg/mL), results are mean values from at least three experiments (triplicate, mean ± SE, with standard errors range 0.1-0.7). (NA) means no activity.

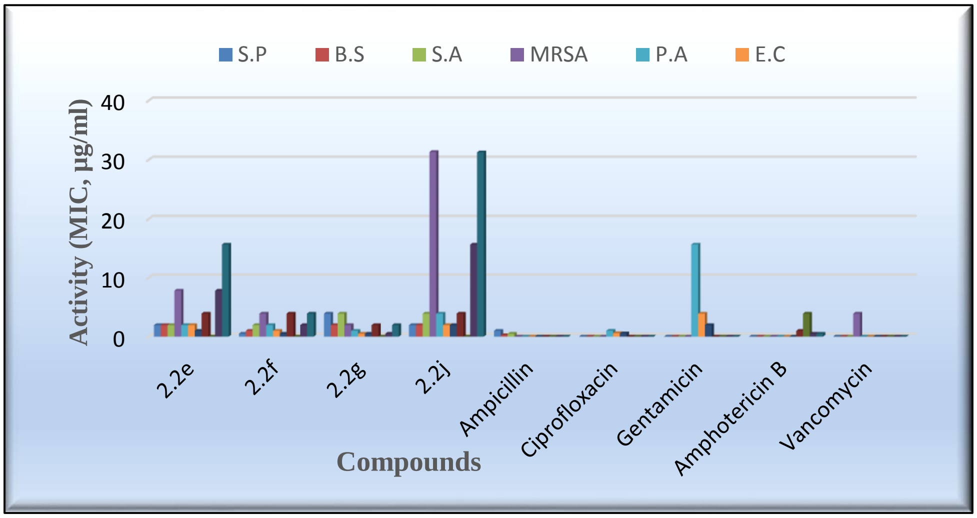


Figure 2.8: Evaluation of minimum inhibitory concentration values (MIC) of compounds.

2.3.2.2 Cytotoxic screening

The *in vitro* cytotoxic activity was performed by MTT assay[121,122] against four human carcinoma cell lines: Human colon carcinoma (HCT-116), human hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7), and adenocarcinomic human alveolar basal epithelial cell (A-549) cell lines. Doxorubicin was used as a positive control, since it has high cytotoxic activity. The inhibitory effects of compounds **2.2(a-j)** on the growth of the four cell lines are shown in **Table 2.4** and **Figure 2.9**. All compounds showed comparable cytotoxicity to the reference drug. Tested compounds exhibited good IC_{50} ranging from 2.9 to 11 µg/mL against HCT-116, while they displayed more inhibitory effects against MCF-7 cell line by IC_{50} 0.86 µg/mL than the standard drug. Moreover, they showed slightly more activity in case of HepG-2 and A-549 cell lines by IC_{50} 1.5 to 9.3

μg/mL range. Further research might lead to improvement in the inhibitory activity, especially for compound **2.2e** against MCF-7 cell line.

Compounds	**IC_{50} (μg/mL)**			
	HCT-116	**MCF-7**	**HepG-2**	**A-549**
2.2a	7.91	6.14	4.84	5.24
2.2b	3.77	2.41	3.97	2.79
2.2c	15.6	22.2	11.8	8.29
2.2d	8.29	6.03	2.91	6.08
2.2e	2.46	0.86	2.19	1.51
2.2f	2.93	2.67	5.56	3.97
2.2g	4.79	18.1	4.65	2.96
2.2h	9.76	9.54	3.3	5.69
2.2i	11.2	23.6	7.71	7.49
2.2j	3.03	9.97	9.39	5.67
Doxorubicin	0.88	1.02	1.19	0.91

Table 2.4: Cytotoxicity of chromene derivatives against four different cancer cell lines.

Cytotoxicity activity results from at least three experiments (triplicate, mean ±SE, with standard errors range 0.02- 0.7). Cytotoxic effects of compounds on colon, breast, liver, and epithelial cell lines following exposure to different concentrations of compounds, and cell viability was assessed

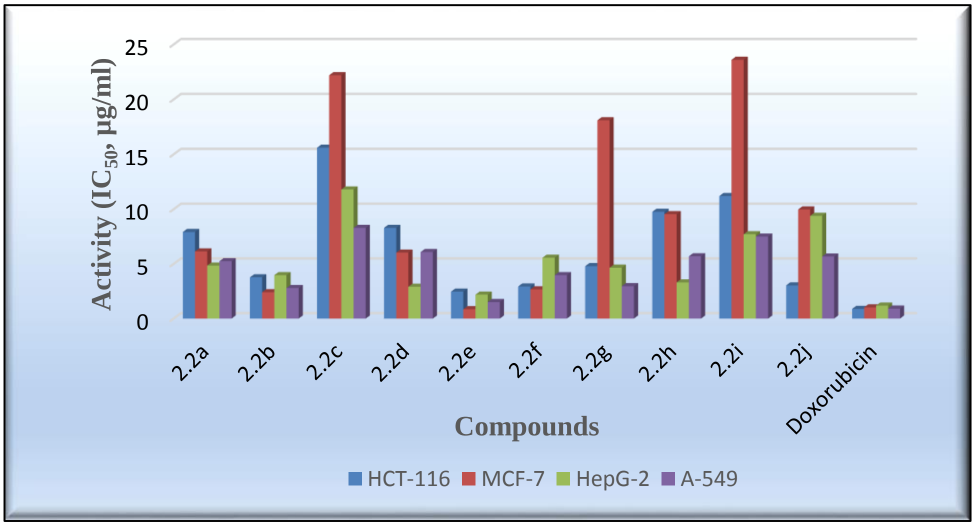


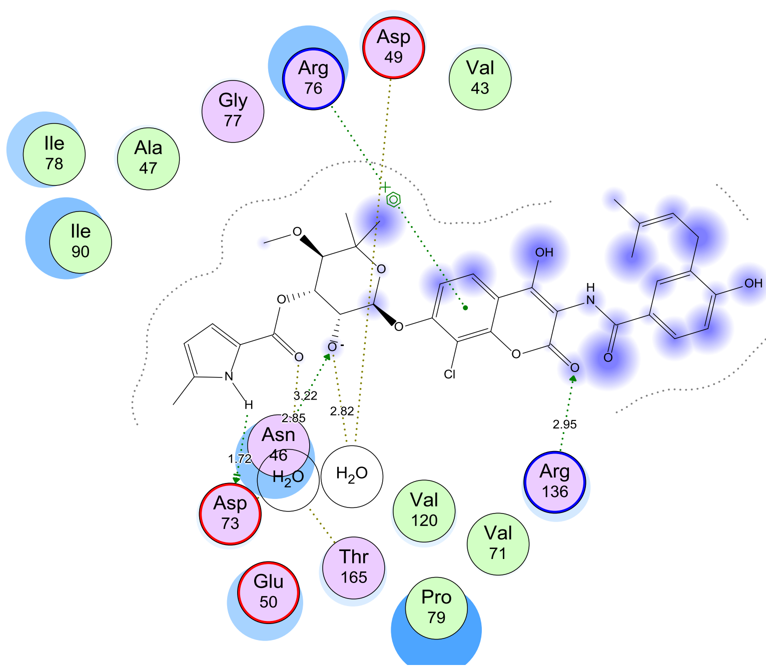
Figure 2.9: Evaluation of cytotoxic activity of target compounds compared to reference drug.

2.3.3 Computational studies

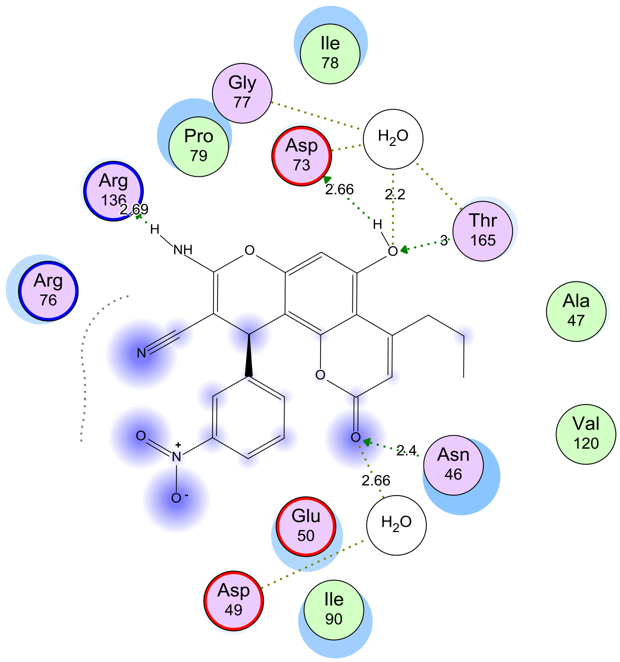
2.3.3.1 Docking studies

Due to the pharmacological profile as the primary target of the investigation of growth inhibition of microorganisms of test compounds and structural similarities for reference ligands, molecular modeling studies using the DNA gyrase B subunit were performed, as it has been reported as a good target for studying inhibitory activity against these bacteria.[123] Docking simulations were performed as a primary step to gain insight into the plausible mechanism of antibacterial activity of the target compounds. *E. coli* topoisomerase II DNA gyrase B (responsible for the supercoiling activity of DNA in bacteria) (PDB code: 1KZN; resolution 2.30 Å) was used by dock module implemented in MOE software. [124,125] For this reason, all compounds were docked into active sites of *E.*

coli topoisomerase II DNA gyrase B complexed with the natural inhibitor clorobiocin. The least energy binding mode of the compounds has been studied. **Figure 2.10** present best docking poses for all investigated chromenes inside topoisomerase II DNA gyrase B binding pocket. The bound clorobiocin drug displayed specific interaction including hydrogen bonding interactions with Asp 73 (1.911 Å), Thr 165 (2.109 Å), Asn 46 (2.034 Å), Arg 76, and Arg 136 (2.071 Å).



Clorobiocin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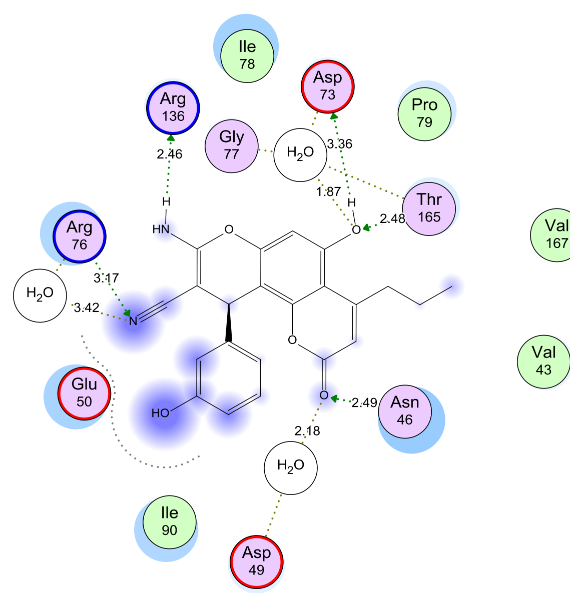
Pro 79
Asp 73
H_2O
Arg 136
2.69
H
NH
O
2.66
2.2
H
O
3
Thr 165
Arg 76
Ala 47
N
O
Val 120
O
N^+
O^-
O
2.4
Asn 46
2.66
Glu 50
H_2O
Asp 49
Ile 90

2.2f


Ile 78
Asp 73
Arg 136
Pro 79
Gly 77
H_2O
3.36
2.46
1.87
Thr 165
H
H
HN
O
2.48
Val 167
Arg 76
O
3.17
H_2O
3.42
N
O
Val 43
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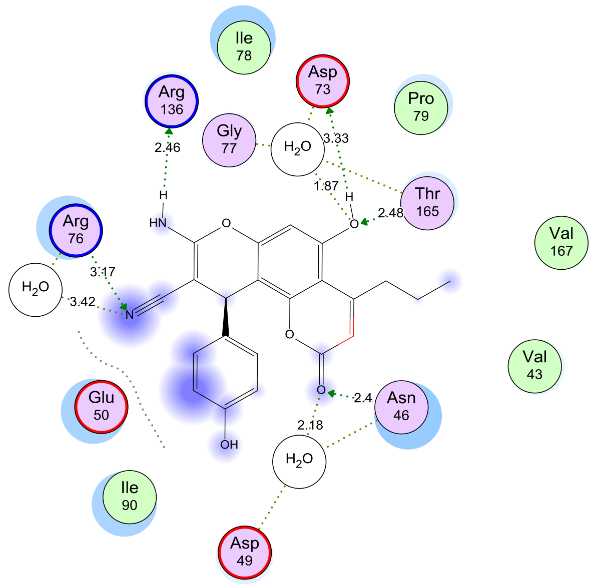
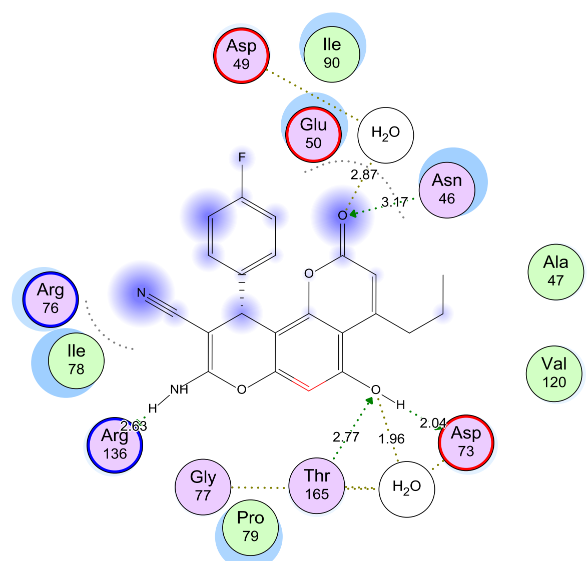
HO
O
2.49
Asn 46
2.18
H_2O
Ile 90
Asp 49

2.2g



2.2i



2.2j

Figure 2.10 2D binding interaction of target compounds clorobiocin**, 2.2f**, **2.2g**, **2.2i** and **2.2j** inside topoisomerase II DNA Gyrase B binding pocket.

Table 2.5: Description of the docking data of the selected target compounds, **2.2f**, **2.2g**, **2.2h** and **2.2j**

Compounds	**Amino acids (Distance Å)**					**Interaction type**	**ΔG (kcal/mol)**
	Asp73	**Thr165**	**Asn46**	**Arg136**	**Arg76**		
2.2f	-OH (2.7)	-OH (3.0)	=O (2.4)	$-NH_2$ (2.7)	-	H-bonding	12.3
2.2g	-OH (3.3)	-OH (2.48)	=O (2.5)	$-NH_2$ (2.5)	-CN (3.2)	H-bonding	13
2.2h	-OH (3.3)	-OH (2.5)	=O (2.4)	$-NH_2$ (2.5)	-CN (3.2)	H-bonding	11.5
2.2j	-OH (2.1)	-OH (2.7)	=O (3.2)	$-NH_2$ (2.4)	-	H-bonding	12.1
Colorobiocin	-NH (1.8)	-OH (H_2O) (3.2)	=O (2.9)	=O (Chr.) (2.9)	Phenyl	H-bonding (Aromatic)	12.5

The data reported in the table are extracted from MOE program showing the corresponding amino acids residues in enzyme pocket, corresponding fragments of ligands, interaction distances, types of interaction, and their binding energy to some selected drugs compared to reference drug.

The results of the docking experiments are presented in **Table 2.5**. The binding map of the compounds in the pocket is explained through four different fragments around the chromene scaffold, 2-C=O, 5-OH, 8-NH_2, and 9-CN. These fragments form stable hydrogen bonding interactions with a panel of corresponding pocket residues: Asn46, Thr165, Asp73, Arg136, and Arg76. The substituted 10-phenyl ring is considered to be the milestone of activity of the compounds via hydrophobic interactions to different residues

Glu50 and Ile90 which contributes considerably to the strength of the binding interactions. Table 2.6 summarizes the nature of 10-phenyl substituents regarding their electronic effect and contribution to the activity. The substitution at position 3 in the ring positively affects the antimicrobial and cytotoxic activity of most compounds, especially those with electron-withdrawing properties such as halogens, nitro, and hydroxyl groups. This has been observed in previous studies[126-128] and is clear with derivatives **2.2(d-g)**, whereas compounds with substituents at 2 or 4 position exhibit moderate to low activity regardless of their types. The bromo derivatives are in most cases highly active, being clearly more effective than chloro and fluoro derivatives, and our results are in agreement with previous studies.[129] Table 2.6 shows the grouping of target compounds with substitution analysis and correlation to their activity. (M) refers to mesomeric effect with positive and negative effects while (I) stands for inductive effect with positive and negative effects.

Table 2.6: Structural and electronic parameters of target compounds and correlation to activity.

<table>
<tr><td></td><td colspan="2">Activity</td><td rowspan="2">Major Electronic effect</td></tr>
<tr><td></td><td>Antibacterial</td><td>Cytotoxicity</td></tr>
<tr><td rowspan="4">High</td><td>2.2e,2.2f,2.2g</td><td>2.2d,2.2e,2.2f</td><td rowspan="4">-I, -M</td></tr>
<tr><td>3-Br,3-NO_2,3-OH</td><td>3-Cl,3-Br,3-NO_2</td></tr>
<tr><td>-I, -M, +M</td><td>-I, -M</td></tr>
<tr><td colspan="2">3 Position</td></tr>
<tr><td rowspan="4">Mild</td><td>2.2a,2.2b,2.2h,2.2i,2.2j</td><td>2.2a,2.2b,2.2h,2.2g</td><td rowspan="4">+I, +M, -I</td></tr>
<tr><td>2-F,2-Cl,4-Butyl,4-OH,4-F</td><td>2-F,2-Cl, 4-Butyl, 3-OH</td></tr>
<tr><td>-I, +I, +M</td><td>+I,+M,-I</td></tr>
<tr><td colspan="2">2,4 Positions</td></tr>
<tr><td rowspan="4">Low</td><td>2.2c,2.2d</td><td>2.2c,2.2i</td><td rowspan="4">-I, +M</td></tr>
<tr><td>2-Br,3-Cl</td><td>2-Br, 4-OH</td></tr>
<tr><td>-I</td><td>-I,+M</td></tr>
<tr><td colspan="2">2,3,4 Positions</td></tr>
</table>

Table shows the grouping of target compounds with substitution analysis and correlation to their activity. (M) refers to mesomeric effect with positive and negative effects while (I) stands for inductive effect with positive and negative effects.

2.4 Conclusions

A series of 8-amino-10-phenyl-5-hydroxy-2-oxo-4-propyl-2*H*, 10*H*-pyrano[2,3-*f*] chromene-9-carbonitrile derivatives **2.2(a-j)** were synthesized by condensation of benzaldehyde derivatives and malononitrile in an ethanolic piperidine (Knoevenagal condensation) followed by the electrophilic substitution step of 5,7-dihydroxy-4-propyl-chromen-2-one (Michael addition adduct). All synthesized compounds were characterized by NMR. Fluorescence of the new compounds showed emission peaks at around 450 nm with good quantum yields. These novel compounds have potent antibacterial, antifungal, and antitumor activities compared to reference drugs. Analysis of the mode of action through docking experiments and structure-activity relationship analysis was reported.

2.5 Experimental Section

2.5.1 Materials and Instrumentation

Chemicals and solvents were purchased from Sigma-Aldrich (Canada) and Alfa Aesar, and were used as received. Compound **2.1** was purchased from Sigma-Aldrich (Canada). Melting points were determined in open capillaries using an electrothermal apparatus and are uncorrected. The progress of the reactions was monitored using thin layer chromatography (TLC) on Merck silica gel 60 F254 plates. Infrared (IR) spectra were recorded using Bruker Alpha FT-IR Spectrometer on pressed KBr pellets. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 300 MHz and at 75 MHz, respectively, on a Bruker Avance Spectrometer in DMSO-d_6 with tetramethylsilane as internal standard. The high vacuum for overnight and heat gun were used to removed residual water from the compounds. Elemental analyses for C, H and N were performed using an Exeter Analytical, Inc. CE-440 Elemental Analyzer. Fluorescence data were acquired on Photon Technology International Quantum Master 400 spectrofluorometer.

The quantum yield of the samples was measurement in 9,10-diphenylanthracene. UV-vis absorption measurements were performed using a HP8543 UV-vis spectrophotometer.

2.5.2 Biological Studies

2.5.2.1 *Antimicrobial Screening*

The microorganism inoculums were uniformly spread using sterile cotton swabs on a sterile Petri dish malt extract agar (for fungi) and nutrient agar (for bacteria). One hundred cubic millimeters of each sample was added to each well (10-mm-diameter holes were cut in the agar gel, 20 mm apart from one another). The systems were incubated for 24–48 hr. at 37 ^{o}C (for bacteria) and at 28 ^{o}C (for fungi). After incubation, microorganism growth was observed. Inhibition zones of the bacterial and fungal growth were measured in millimeters. Tests were performed in triplicate. [113,114]

2.5.2.2 *Cytotoxic Screening*

Human colon carcinoma (HCT-116), human hepatocellular carcinoma (HEPG-2), adenocarcinomic human alveolar basal epithelial cell (A-549), and human breast adenocarcinoma (MCF-7) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/mL gentamycin. The cells were maintained at 37 ºC in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. Potential cytotoxicity of the compounds was evaluated on tumor cells using the method of Gangadevi and Muthumary.[130] The cells were grown as monolayers in growth RPMI-1640. The monolayers of 104 cells adhered at the bottom of the wells in a 96-well microliter plate incubated for 24 h at 37 ºC in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M pH 7.2) and simultaneously the cells were treated with 100 µL from different dilutions of tested sample in fresh maintenance medium and incubated at 37 ºC. A control of untreated cells was made in the absence of tested sample. Positive controls containing doxorubicin were also tested as a reference drug for comparison. Six wells were used for each concentration of the test sample. Every 24-hour observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and the absorbance read at 590 nm using a microplate reader (SunRise, TECAN, Inc, USA) through mixing[121,131]. The absorbance values from untreated cells were considered as 100% proliferation. The number of viable cells was determined using microplate reader as previously mentioned and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration was plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50 % inhibitory concentration

(IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

2.5.3 Molecular modeling

The newly synthesized compounds were docked into the crystal structure of *E. coli* topoisomerase II DNA gyrase B (PDB code 1KZN). The MOE software[124] was used for all docking calculations. The MOE tools package was employed to generate the docking input files and to analyze the docking results. All non-polar hydrogens, clorobiocin, and crystal water molecules were removed prior to the calculations, the protonation of the enzyme was carried out and the structure was energy minimized. In each case, 100 docked structures were generated using genetic algorithm searches. The 3D structures of new synthesized compounds were drawn in MOE and the protonation of ligands were carried out. The energy of compounds was minimized up to 0.05 gradient using GBVI/WSA dG force field. These data were saved in database as input file MOE. Heavy atom comparison root means square deviations (RMSD values) were calculated and initial ligand binding modes were plotted. Protein-ligand interaction plots were generated using MOE 2012.10. Quantum mechanical calculations and surface molecular orbitals were generated by simulation module in MOE software.

2.5.4 Synthesis

2.5.4.1 General procedure for the synthesis of 8-amino-10-phenyl-5-hydroxy-2 oxo-4-propyl-2H, 10H-pyrano[2,3-f] chromene-9-carbonitrile derivatives.

Following similar procedure to the synthesis of chromenes,[132] aryl aldehyde (2.3 mmol) was added to a solution containing compound 2.1 (2.3 mmol) in 5 mL of ethanol and malononitrile (2.3 mmol), along with few drops of piperidine. The reaction mixture was stirred at reflux. After completion of the reaction (monitored by TLC), the mixture was kept at room temperature and the formed solid product was collected by filtration and washed with ethanol and hexane to yield **2.2(a-j)**.

2.5.4.2 8-amino-10-(2-fluorophenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2a)

White solid (0.87g, 97%), m.p. 225°C; IR (KBr) cm^{-1}: 3483(OH), 3403 (NH_2), 3055 (Ar-H), 2966- 2871 (CH), 2199 (CN), 1697 (C=O); ^1H NMR (300 MHz, DMSO) δ 10.98 (s, 1H, OH), 7.27 – 7.22 (m, 1H, Ar-H), 7.16 – 7.09 (m, 3H, Ar-H), 6.97 (s, 2H, NH_2), 6.50 (s, 1H, H3), 6.07 (s, 1H, H6), 4.91 (s, 1H, H10), 3.16-3.09 (m, 1H, CH_2), 2.93-2.86 (m, 1H, CH_2), 1.68-1.59 (m, 2H, CH_2), 0.97 (t, J = 7.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 160.47 (C=O), 160.24, 159.19, 158.28, 157.48, 155.71, 148.26, 132.70 (Ar-C), 130.68, 129.41, 125.42 (Ar-CH), 120.73 (Ar-C), 116.16 (Ar-CH), 111.89 (Ar-CH-3), 108.13 (CN), 101.71 (Ar-C), 99.68 (Ar-CH-6), 57.14 (C-8), 38.27 (CH_2), 31.72 (CH-10), 23.59 (CH_2), 14.15 (CH_3); Anal. Calcd for $C_{22}H_{17}N_2O_4F$: C, 67.34; H, 4.37; N, 7.14. Found: C, 67.05; H, 4.01; N, 7.03.

2.5.4.3 8-amino-10-(2-chlorophenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene -9-carbonitrile (2.2b)

White solid (0.78g, 83%), m.p. 266°C; IR (KBr) cm^{-1}: 3491 (OH), 3405 (NH_2), 3063 (Ar-H), 2971- 2866 (CH), 2193 (CN), 1725 (C=O); ^{1}H NMR (300 MHz, DMSO) δ 10.86 (br. s, 1H, OH), 7.40 - 7.37 (m, 1H, Ar-H), 7.27 – 7.17 (m, 2H, Ar-H), 7.05 – 7.02 (m, 1H, Ar-H), 6.95 (s, 2H, NH_2), 6.43 (s, 1H, H3), 6.06 (s, 1H, H6), 5.16 (s, 1H, H10), 3.14-3.06 (m, 1H, CH_2), 2.96- 2.89 (m, 1H, CH_2), 1.71-1.56 (m, 2H, CH_2), 0.97 (t, *J* = 7.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 160.31 (C=O), 158.95, 157.58, 155.86, 148.28, 143.17, 132.80 (Ar-C), 130.97, 130.23, 129.06, 128.46 (Ar-CH), 120.38 (Ar-C), 111.70 (Ar-CH-3), 108.40 (CN), 101.42 (Ar-C), 99.89 (Ar-CH-6), 57.15 (C-8), 38.06 (CH_2), 34.82 (CH-10), 22.92 (CH_2), 14.29 (CH_3); Anal. Calcd for $C_{22}H_{17}N_2O_4Cl$: C, 64.63; H, 4.19; N, 6.85. Found: C, 64.86; H, 4.52; N, 6.52.

2.5.4.4 8-amino-10-(2-bromophenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2c)

Yellowish white solid (0.99g, 95%), m.p. 282°C; IR (KBr) cm^{-1}: 3501 (OH), 3403 (NH_2), 3068 (Ar-H), 2962- 2869 (CH), 2200 (CN), 1723 (C=O); ^{1}H NMR (300 MHz, DMSO) δ 7.56 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.30-7.26 (m, 1H, Ar-H), 7.13-7.08 (m, 1H, Ar-H), 7.09 (d, *J* = 8.0 Hz, 1H, Ar-H), 6.93 (br. s, 2H, NH2), 6.39 (s, 1H, 3), 6.05 (s, 1H, Ar-H6), 5.18 (s, 1H, 10), 3.14-3.05 (m, 1H, CH_2), 2.97-2.90 (m, 1H, CH_2), 1.68 – 1.61 (m, 2H, CH_2), 0.98 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 160.30 (C=O), 159.69, 157.57, 156.00, 148.18, 145.06 (Ar-C), 133.33, 131.18, 128.83, 123.36 (Ar-CH), 120.33 (Ar-C), 111.23 (Ar-CH-3), 108.69 (CN), 101.07 (Ar-C), 99.51 (Ar-CH-6), 57.70 (C-8), 38.23 (CH_2), 36.65 (CH-10), 22.98 (CH_2), 14.17 (CH_3); Anal. Calcd for $C_{22}H_{17}N_2O_4Br$: C, 58.29; H, 3.78; N, 6.18. Found: C, 58.38; H, 4.16; N, 5.99.

2.5.4.5 8-amino-10-(3-chlorophenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2d)

Yellowish white solid (0.74g, 79%), m.p. 275°C; IR (KBr) cm^{-1}: 3502 (OH), 3409 (NH_2), 2953- 2875 (CH), 2187 (CN), 1704 (C=O); ^1H NMR (300 MHz, DMSO) δ 11.09 (s, 1H, OH), 7.34 - 7.28 (m, 2H, Ar-H), 7.16-7.07 (m, 2H, Ar-H), 7.05 (s, 2H, NH_2), 6.53 (s, 1H, H3), 6.08 (s, 1H, H6), 4.69 (s, 1H, H10), 3.11-3.04 (m, 1H, CH_2), 2.98-2.91 (m, 1H, CH_2), 1.65-1.58 (m, 2H, CH_2), 0.97 (t, *J* = 7.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 160.47 (C=O), 158.42, 15803, 155.62, 148.14, 148.03, 133.79 (Ar-C), 131.77, 127.53, 127.50, 126.52 (Ar-CH), 120.65 (Ar-C), 111.76 (Ar-CH-3), 109.13 (CN), 102.26 (Ar-C), 99.84 (Ar-CH-6), 58.00 (C-8), 38.40 (CH_2), 37.19 (CH-10), 23.24 (CH_2), 14.75 (CH_3); Anal. Calcd for $C_{22}H_{17}N_2O_4Cl$: C, 64.63; H, 4.19; N, 6.85. Found: C, 64.99; H, 4.56; N, 6.54.

2.5.4.6 8-amino-10-(3-bromophenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2e)

White solid (0.82g, 80%), m.p. 253°C; IR (KBr) cm^{-1}: 3597 (OH), 3405 (NH_2), 3055 (Ar-H), 2952- 2861 (CH), 2195 (CN), 1721 (C=O); ^1H NMR (300 MHz, DMSO) δ 7.38 (d, *J*

= 8.0 Hz, 1H, Ar-H), 7.30-7.24 (m, 2H, Ar-H), 7.14 (d, *J* = 7.2 Hz 1H, Ar-H), 6.99 (s, 2H, NH_2), 6.34 (s, 1H, H3), 5.96 (s, 1H, H6), 4.66 (s, 1H, H10), 3.12-2.99 (m, 1H, CH_2), 2.97-2.85 (m, 1H, CH_2), 1.65 – 1.58 (m, 2H, CH_2), 0.96 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 160.88 (C=O), 157.69, 156.29, 149.31, 148.11 (Ar-C), 131.94, 130.54, 127.35, 122.76 (Ar-CH), 120.76 (Ar-C), 111.18 (Ar-CH-3), 109.58 (CN), 100.52 (Ar-C), 100.60 (Ar-CH-6), 58.29 (C-8), 38.32 (CH_2), 37.13 (CH-10), 22.95 (CH_2), 14.57 (CH_3); Anal. Calcd for $C_{22}H_{17}N_2O_4Br$: C, 58.29; H, 3.78; N, 6.18. Found: C, 58.69; H, 4.11; N, 6.29.

2.5.4.7 8-amino-10-(3-nitrophenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2f)

White solid (0.90g, 94%), m.p. 226°C; IR (KBr) cm^{-1}: 3495 (OH), 3404 (NH_2), 3067 (Ar-H), 2969- 2878 (CH), 2200 (CN), 1727 (C=O), 1531, 1354 (NO); ^{1}H NMR (300 MHz, DMSO) δ 8.10- 8.06 (m, 1H, Ar-H), 7.98 (br. s, 1H, Ar-H), 7.63 – 7.59 (m, 2H, Ar-H), 7.11 (s, 2H, NH_2), 6.46 (s, 1H, H3), 6.04 (s, 1H, H6), 4.87 (s, 1H, H10), 3.14-3.05 (m, 1H, CH_2), 2.97-2.87 (m, 1H, CH_2), 1.67 – 1.60 (m, 2H, CH_2), 0.97 (t, *J* = 7.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 160.78 (Ar-C), 160.28 (C=O), 159.22, 157.42, 156.05, 148.78, 148.28, 147.91 (Ar-C), 134.86, 131.01, 122.74, 122.31 (Ar-CH), 120.69 (Ar-C), 111.62 (Ar-CH-3), 108.57 (CN), 102.01 (Ar-C), 99.92 (Ar-CH-6), 58.07 (C-8), 38.45 (CH_2), 37.14 (CH-10), 23.54 (CH_2), 14.91 (CH_3); Anal. Calcd for $C_{22}H_{17}N_3O_6$: C, 63.01; H, 4.09; N, 10.02. Found: C, 63.32; H, 4.34; N, 9.69.

2.5.4.8 8-amino-10-(3-hydroxyphenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2g)

White solid (0.75g, 84%), m.p. 213°C; IR (KBr) cm^{-1}: 3493 (OH), 3399 (NH_2), 3067 (Ar-H), 2942- 2865 (CH), 2181 (CN), 1711 (C=O); ^1H NMR (300 MHz, DMSO) δ 7.02 (t, *J* = 8.4 Hz, 1H, Ar-H), 6.89 (s, 2H, NH_2), 6.60-6.52 (m, 3H, Ar-H), 6.38 (s, 1H, 3), 5.96 (s, 1H, Ar-H6), 4.54 (s, 1H, 10), 3.08-2.99 (m, 1H, CH_2), 2.86-2.79 (m, 1H, CH_2), 1.65–1.58 (m, 2H, CH_2), 0.97 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 161.10 (C=O), 160.67, 158.21, 157.52, 155.94, 148.25, 147.66 (Ar-C), 130.11 (Ar-CH), 121.04 (Ar-C), 118.48, 114.74, 114.15 (Ar-CH), 110.50 (Ar-CH-3), 109.71 (CN), 100.74 (Ar-C), 99.85 (Ar-CH-6), 58.95 (C-8), 38.20 (CH_2), 36.93 (CH-10), 23.02 (CH_2), 14.18 (CH_3); Anal. Calcd for $C_{22}H_{18}N_2O_5$: C, 67.69; H, 4.65; N, 7.18. Found: C, 67.91; H, 4.90; N, 7.66.

2.5.4.9 8-amino-10-(4-butylphenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2h)

White solid (0.87g, 88%), m.p. 235°C; IR (KBr) cm^{-1}: 3499 (OH), 3404 (NH_2), 3067 (Ar-H), 2958- 2859 (CH), 2196 (CN), 1722 (C=O); ^1H NMR (300 MHz, DMSO) δ 11.02 (br.s, 1H, OH), 7.09 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.01 (d, *J* = 8.1 Hz, 2H, Ar-H), 6.96 (s, 2H, NH_2), 6.53 (s, 1H, H3), 6.06 (s, 1H, H6), 4.61 (s, 1H, H10), 3.4-3.2 (m, 2H, CH_2), 3.16- 3.06 (m, 1H, CH_2), 2.96 – 2.87 (m, 1H, CH_2), 1.62 (q, *J* = 7.2 Hz, 2H, CH_2), 1.52 (q, *J* = 7.2 Hz, 2H, CH_2), 1.32-1.27 (m, 2H, CH_2), 0.97 (t, *J* = 7.4 Hz, 3H, CH_3), 0.87 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 160.50 (C=O), 160.24, 158.25, 157.49, 155.50, 148.05, 143.26, 141.42 (Ar-C), 129.23, 127.57 (Ar-CH), 120.94 (Ar-C), 111.94 (Ar-CH-3), 109.87 (CN), 101.88 (Ar-C), 99.81 (Ar-CH-6), 58.84 (C-8), 37.99 (CH_2), 36.65 (CH-10), 35.32 (CH_2), 34.09 (CH_2), 31.60 (CH_2), 22.69 (CH_2), 14.62 (CH_3), 14.14 (CH_3); Anal. Calcd for $C_{26}H_{26}N_2O_4$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.57; H, 6.24; N, 6.30.

2.5.4.10 8-amino-10-(4-hydroxyphenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyra-non [2, 3-f] chromene-9-carbonitrile (2.2i)

White solid (0.71g, 81%), m.p. 209°C; IR (KBr) cm^{-1}: 3495 (OH), 3400 (NH_2), 2958-2867 (CH), 2194 (CN), 1717 (C=O); ^{1}H NMR (300 MHz, DMSO) δ 10.94 (s, 1H, OH), 9.29 (s, 1H, OH), 6.93 (d, J = 8.4 Hz, 2H, Ar-H), 6.90 (s, 2H, NH_2), 6.66 (d, J = 8.4 Hz, 2H, Ar-H), 6.52 (s, 1H, H3), 6.09 (s, 1H, H6), 4.53 (s, 1H, H10), 3.13-3.05 (m, 1H, CH_2), 2.95-2.86 (m, 1H, CH_2), 1.66-1.59 (m, 2H, CH_2), 0.97 (t, J = 7.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 161.11 (C=O), 160.86, 157.95, 156.67, 147.96, 137.23, 121.53 (Ar-C), 128.79, 115.72 (Ar-CH), 111.58 (CN), 107.74 (Ar-CH-3), 100.34 (Ar-CH-6), 98.10 (Ar-C), 59.48 (C-8), 38.18 (CH_2), 36.37 (CH-10), 23.11 (CH_2), 14.27 (CH_3); Anal. Calcd for $C_{22}H_{18}N_2O_5$: C, 67.69; H, 4.65; N, 7.18. Found: C, 67.26; H, 4.34; N, 7.50.

2.5.4.11 8-amino-10-(4-fluorophenyl)-5-hydroxy-2-oxo-4-propyl-2H,10H-pyrano [2,3-f] chromene-9-carbonitrile (2.2j)

Yellowish white solid (0.71g, 79%), m.p. 197.2°C; IR (KBr) cm^{-1}: 3403 (OH), 3398 (NH_2), 3055 (Ar-H), 2966- 2932 (CH), 2189 (CN), 1721 (C=O); ^{1}H NMR (300 MHz, DMSO) δ 7.17- 7.11 (m, 4H, Ar-H), 6.97 (s, 2H, NH_2), 6.48 (s, 1H, H3), 6.04 (s, 1H, H6), 4.66 (s, 1H, H10), 3.11–3.04 (m, 1H, CH_2), 2.93-2.89 (m, 1H, CH_2), 1.66 – 1.58 (m, 2H, CH_2), 0.97 (t, J = 7.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 163.38 (C=O), 160.45, 158.67, 157.52, 155.68, 147.90, 142.28 (Ar-C), 129.77, 129.61 (Ar-CH), 120.85 (Ar-C), 116.21, 115.94 (Ar-CH), 111.71 (Ar-CH-3), 109.44 (CN), 101.75 (Ar-C), 99.84 (Ar-CH-6), 58.62 (C-8), 38.21 (CH_2), 36.50 (CH-10), 22.84 (CH_2), 14.30 (CH_3); Anal. Calcd for $C_{22}H_{17}N_2O_4F$: C, 67.34; H, 4.37; N, 7.14. Found: C, 66.98; H, 4.61; N, 6.94.

Chapter Three: Synthesis, Antimicrobial, Cytotoxicity Behavior and Structure Activity Relationships of Chromene Based Flavanone Moiety.

3.1 Synopsis

Several new 8-amino-10-phenyl-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-diydro-2*H*, 10*H*-pyrano [2,3-*f*] chromene derivatives **3.2(a-g)** were synthesized in good yields. The structures of these derivatives were established on the basis of IR, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and elemental analysis. All new synthesized compounds were evaluated for in vitro antimicrobial and cytotoxicity activities. Their antimicrobial activity was investigated and tested against seven human pathogen Gram-positive and Gram-negative bacteria: *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, four fungi: *Aspergillus niger, Aspergillus flavus, Fusarium oxisporum, Rhizopus stolonifer* and one mycobacterium, *Mycobacterium tuberculosis*, using agar well diffusion method and minimum inhibitory concentrations were reported. All of the new derivatives exhibited significant potent antimicrobial activities against most bacterial strains compared to reference drugs. The results of antibacterial assay revealed that the compounds **3.2a, 3.2b** and **3.2(e-g)** exhibited the highest inhibitory activity against *Pseudomonas aeruginosa* and *E. coli* by IZ range 18.3 to 24.6 mm and MIC 0.49 to 3.9 µg/ml compared to reference drugs. Compounds **3.2a**, **3.2b** and **3.2e** were found to be more effective against *Salmonella typhimurium* by IZ 23.4-26.4 mm.

The antibacterial activity of most compounds was found to be comparably active to reference drugs against Gram-positive bacteria. Compound **3.2c** did not show any antimicrobial activity against all the tested bacteria, fungi and TB. Moreover, the cytotoxic activity was also evaluated against four different human carcinoma cell lines: human colon carcinoma (HCT-116), human hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7), and adenocarcinoma human alveolar basal epithelial cell (A-549) cell lines, and exhibited good cell growth inhibitory activity against HCT-116 cell line (IC_{50}= 1.08-1.48 μg/ml). The molecular modeling results showed binding interaction of synthesized compounds **3.2a**, **3.2b**, **3.2d**, **3.2e** and kaempferol as reference drug in the active site of Gyres B.

3.2 Introduction

Substantial increasing of resistance of microorganisms against used antimicrobial agents is one of major concern among scientists and clinicians worldwide. Moreover, the pathogenic viruses, bacteria, fungi, and protozoa become more and more difficult to treat with the already existing drugs.[133] To overcome the drawbacks of the current antimicrobial drugs and to obtain more efficacious drugs, an antimicrobial drug having a novel mode of action should be developed.[134] The abuse of synthetic antibiotics has contributed to the increased incidence of bacterial resistance to available antibacterial agents, resulting in an urgent need for natural antimicrobials.[135]

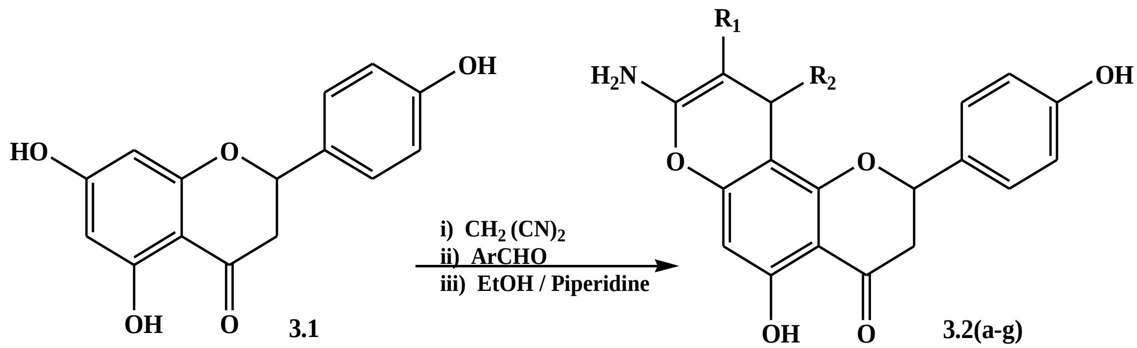
Plant-derived flavonoids are a large group of naturally occurring phenyl chromones widely distributed in edible plants and found in fruits, vegetables, tea, and wine.[136,137] They have been shown to have a wide range of biological activities, including antiallergic, antibacterial, antidiabetic, anti-inflammatory, antiviral, antiproliferative, antimutagenic, antithrombotic, anticarcinogenic, hepatoprotective, oestrogenic, insecticidal, and antioxidant activities.[138,139] The antibacterial activities of flavonoids have been reported to be related to their chemical structures.[139-142] However, the quantitative structure activity relationship (QSAR) for flavonoids as antibacterial agents is gaining interest by quantitatively correlating the molecular structures or properties with variation in biological activity.[143,144] Most of the interesting pharmacological properties of flavonoids is the anticancer activity[145], which reported different mechanisms of the effects of flavonoids aiding in the prevention of cancer growth through their ability to work as anti-oxidants,[69-71] enzyme inhibitors[146] and growth regulators.[147] Flavanones, 2-phenylchroman-4-one, belong to the family of flavonoids, many of which are produced as secondary metabolites in the plant kingdom. They have three-ring skeletons, C6-C3-C6, and the rings are referred to as A-, C-, and B-rings, respectively. Their functional groups are attached to the main skeleton through oxygen or carbon linkages.[148] The biological activities of flavonoids depend on the degree of condensation in their structures and the position and number of substitutions, such as hydroxy groups, glucosides, isoprenyl units, homodimers, and heterodimers.[149]

Flavanones compared to flavanone, 2-phenyl-4H-chromen-4-one, have more molecular flexibility due to the absence of a carbon–carbon double bond in the C-ring. In our previous studies, we found that methoxy or hydroxy substituent at C-7 position of flavanone resulted in better inhibitory effects on HCT116 human colon cancer cell lines.[150] Based on this result, we tried to design flavanone derivatives with bulkier substituents at C-7 position and elucidate their inhibitory effects. Since the anti-cancer activity of naringenin, 4',5,7-trihydroxyflavanone **3.1** against colon cancer cells has been reported, several flavanone derivatives were designed from naringenin.[151] In addition, it was found that the naringenin plays a key role as an estrogenic substance in humans and as an endogenous regulator in plants.[152] Various flavonoid derivatives modified at C-7 position have been reported, but in flavanone, especially naringenin, derivatives modified at position 7 have rarely been studied.[153, 154] Naringenin derivatives designed in this study have a common moiety formed between 7-OH and 8-H positions resulting NH_2-CC-CN fragment which may affect their biological activity.

3.3 Results and Discussion

3.3.1 Synthesis and Characterization

All compounds **3.2(a-g)** were synthesized according to the steps outline in **Scheme 3.1**. The chromene derivatives based on 4`,5,7-trihydroxyflavanone **3.1** were prepared by condensation between 4`,5,7-trihydroxyflavanone, malononitrile or ethylcyanoacetate and aromatic aldehydes in the presence of piperidine in ethanol under reflux **(Table 3.1)**.

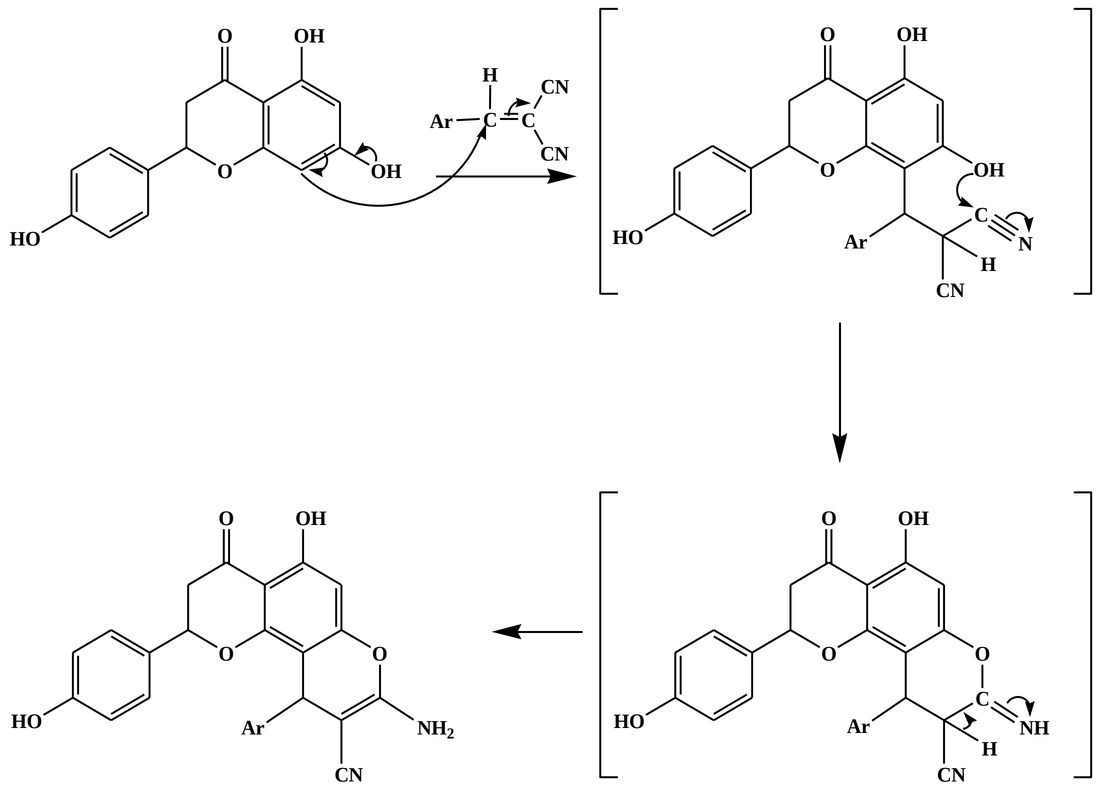


Scheme 3.1: Synthesis of 8-amino-10-phenyl-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-diydro-2*H*,10*H*-pyrano[2,3-*f*] chromene derivatives **3.2(a-g)**.

Compound No.	R_1	R_2	Yield%
3.2a	CN	2-ClC_6H_5	89.51
3.2b	CN	2-FC_6H_5	71.51
3.2c	CN	3-BrC_6H_5	77.42
3.2d	CN	4-$C(CH_3)_3C_6H_5$	84.36
3.2e	CN	C_4H_4S	88.31
3.2f	CN	4-FC_6H_5	68.35
3.2g	COOEt	3-$NO_2C_6H_5$	74.58

Table 3.1: The yields of new synthesized derivatives **3.2(a-g).**

The first step of this multicomponent process begins with Knoevenagel condensation and then followed by Michael addition adducts[113], as shown in the following **Scheme 3.2**.



Scheme 3.2: General synthesis and mechanistic pathway of chromene molecules **3.2(a-g)**

The newly synthesized derivatives were confirmed by the various analytical techniques such as FT-IR, ^{1}H NMR, ^{13}C NMR, COSY, HMBC, HSQC and elemental analysis. FT-IR spectroscopy showed characteristic absorption bands between 2178 and 2207 for the CN groups while the NH_2 stretches were in the range of 3381-3402 cm^{-1}. Moreover, the C=O group displayed bands at 1634-1686 cm^{-1} and the OH bands appeared at 3477-3634 cm^{-1}. In the ^{1}H NMR spectra of synthesized derivatives **3.2(a-g)** were obtained in DMSO-d_6, the H2 signal showed as a doublet of doublets around 5.07-5.64 ppm. The resonances of the diastereotopic H3ax and H3eq protons appeared approximately at 3.34-3.51 and 2.72-2.83 ppm as two doublets of doublets. The H3ax and H3eq of chromane ring are coupled with a constant of 17.1-17.6 Hz related to the geminal coupling. The value of coupling constant between H2 and H3ax is too large (J=12.2-13.5 Hz) which can only arise from a trans-diaxial coupling, thus H2 is axial and the aryl group was linked to H2 has equatorial orientation. As anticipated, the hydroxyl group linked to C5 appeared as a singlet set at 11.9-12.5 ppm and the C4`-OH occurred approximately at 9.62-9.63 ppm, while the H10 pyran showed signals at 4.46-5.08 ppm and amine protons resonated at 7.03-7.79 ppm. The singlet at 6.21-6.27 ppm is corresponding to H6, while aromatic protons resonate further downfield between 6.74 and 8.08 ppm. **Figure 3.1** shows one example of the proton NMR for compound **3.2a**. In compound **3.2g** spectrum, the methyl protons appeared at 0.98 ppm as triplet, while -O**CH₂**CH₃ showed at 3.81 to 4.03 ppm.

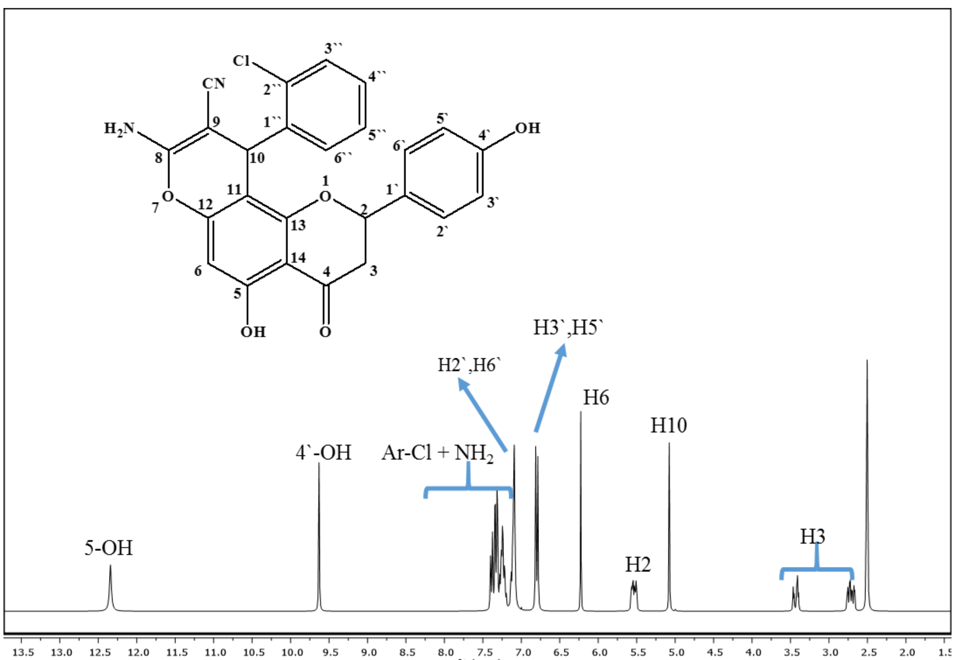


Figure 3.1: ^{1}H NMR of Compound **3.2a**

The ^{13}C NMR spectra of the new molecules showed signals at 78.8-80.2 ppm and 41.7-43.4 ppm represent C-2 and C-3 respectively, C-2 being assigned the lower field signal because it is oxygenated, while C-6 signal showed at 94.8-96.8 ppm. The B ring carbons, C-2`and C-6` presented at 128.5-129.5 ppm, while C-3` and C-5` showed at 115.9-116.1 ppm. The C-4` which is connected to hydroxyl group appeared at 157.9-158.9 ppm and the signal at 128.4-132.8 ppm is ascribed to C-1`. The signal at 30.3-36.2 ppm corresponds to C-10 of the pyran ring, while the quaternary carbon C-8 that is attached to amine group appeared in the range of 159.3-161.9 ppm. The cyanide functionality resonated further downfield at 119.7-121.0 ppm and the aromatic carbons showed signals between 162.9 and 102.8 ppm, with an additional signal for C=O of flavanone moiety at 198.0-198.9 ppm **(Figure 3.2)** The carbon atom attached to the methyl group in compound **4.2g** resonated at 15.0 ppm, while the -O**CH_2**CH_3 carbons appeared at 59.8 ppm and signal for (C=O ester) showed at 168.7 ppm.

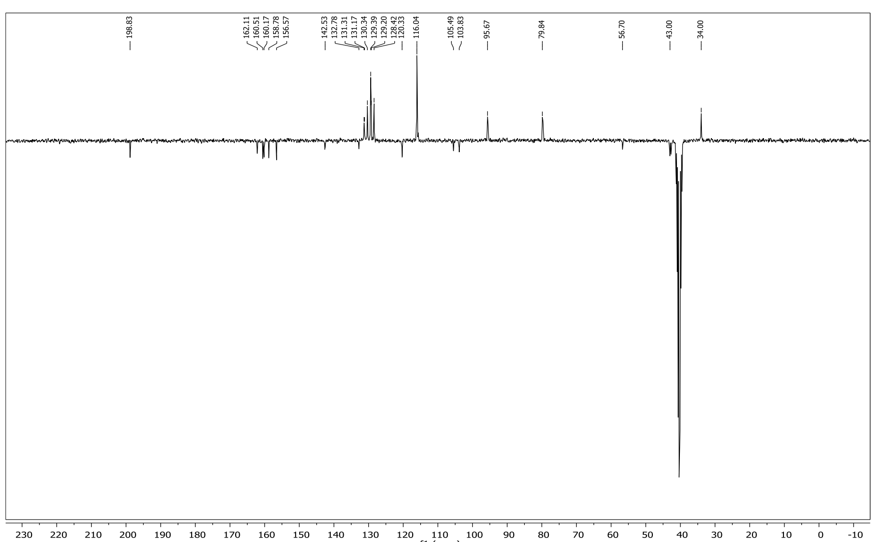


Figure 3.2: DEPTQ135 ^{13}C NMR of Compound **3.2a**

3.3.2 Biological Screening

3.3.2.1 Antimicrobial Screening

The new synthesized compounds **3.2(a-g**) were screened in vitro for their antibacterial, antifungal, and antimycobacterium activities via agar diffusion well method.[117] The activity of the synthesized compounds was tested against four Gram-positive bacteria including *Streptococcus pneumoniae* (RCMB 010010), *Bacillus subtilis* (RCMB 010067), *Staphylococcus aureus* (RCMB 000106), and *Methicillin-Resistant Staphylococcus aureus* (MRSA 2658 RCMB), three Gram-negative bacteria including *Pseudomonas aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052), and *Salmonella typhimurium* (RCMB 000106), four fungi including *Aspergillus fumigatus* (RCMB 02568), *Syncephalastrum racemosum* (RCMB 05922*), Geotricum candidum* (RCMB 05097), and *Candida albicans* (RCMB 05036), and finally, against *Mycobacterium tuberculosis* (RCMB 010094-8). Ampicillin, Gentamicin, amphotericin B, vancomycin, and isoniazid were used as control drugs.[119, 120] The inhibition zones and minimum inhibitory concentrations (MIC) were determined by serial dilution method. The values of inhibition zone (IZ) and minimum inhibitory concentrations (MIC) of the target compounds against P.A and *E. coli* revealed that derivatives **3.2a-b** and **3.2(d-g)** showed the highest inhibitory activity by IZ range 18.3 to 24.6 mm and MIC 0.49 to 3.9 µg/ml compared to reference drugs, while the results of inhibition zone (IZ) and minimum inhibitory concentrations (MIC) were displayed in **Table 3.2, 3.3** and presented in **Figure 3.3, 3.4**. Compounds **3.2a**, **3.2b** and **3.2e** were found to be more effective against S.T by IZ 23.4-26.4 mm. The antibacterial activity of most compounds were found to be comparably active to reference drugs against gram-positive bacteria, having MIC range 0.49 to 3.9 µg/ml. Compounds **3.2b** and **3.2e** exhibited mild inhibitory activity against MRSA by IZ 21.3-20.6 mm and MIC 1.95-3.9 µg/ml. Most of the synthesized derivatives were slightly active against the TB and fungal species. Compound **3.2e** exhibited moderately active to isoniazid. On the other hand, compound **3.2c** did not show any antimicrobial activity against all the tested bacteria, fungi and TB. In general, the antimicrobial activity of the new derivatives showed more potency than the reference drugs against Gram-negative bacteria and mild activity towards Gram-positive bacteria, fungi, and TB.

Compounds	**Inhibition Zone Diameter (mm)**											
	Gram-positive				**Gram-negative**			**Fungi**				**TB**
	S.P	**B.S**	**S.T**	**MRSA**	**P.A**	**E.C**	**S.T**	**A.F**	**S.R**	**G.C**	**C.A**	
3.2a	21.3	23.2	22.4	18.6	20.3	22.4	23.4	21.3	NA	23.1	20.4	53.1
3.2b	22.6	26.4	24.6	20.6	22.6	24.6	26.3	23.4	NA	25.2	22.4	72.3
3.2c	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3.2d	18.6	21.3	21.5	17.9	19.3	21.4	21.9	18.3	NA	20.9	20.1	56.3
3.2e	23.1	26.4	24.2	21.3	21.3	24.2	26.4	22.3	NA	26.4	21.3	80.1
3.2f	17.2	18.9	20.3	16.8	18.3	20.3	21.5	19.6	NA	18.4	18.1	41.2
3.2g	19.4	21.5	21.9	18.4	18.9	20.1	21.2	19.3	NA	21.2	20.4	67.3
Ampicillin	23.8	32.4	26.2	-	-	-	-	-	-	-	-	-
Gentamicin	-	-	-	-	17.3	19.9	22.3	-	-	-	-	-
Ciprofloxacin	-	-	-	-	23.4	26.2	27.4	-	-	-	-	-
Amphotericin B	-	-	-	-	-	-	-	23.7	19.7	28.7	25.4	-
Vancomycin	-	-	-	20.3	-	-	-	-	-	-	-	-
Isoniazid	-	-	-	-	-	-	-	-	-	-	-	83.2

Table 3.2: Antimicrobial activity of synthetic compounds (IZ, diameter (mm)) (1 mg/mL).

Mean zone of inhibition in mm from at least three experiments; (triplicate, mean ± SE, with standard errors range 0.01- 0.8). (NA) means no activity. S.P (*Streptococcus pneumoniae*), B.S (*Bacillus subtilis*), P.A (*Pseudomonas aeruginosa*), E.C (*Escherichia coli*), S.T (*Salmonella typhimurium*), A.F *Aspergillus fumigatus* (RCMB 02568), G.C *Geotricum candidum* (RCMB 05097), S.R *Syncephalastrum racemosum* (RCMB 05922), and C.A *Candida albicans* (RCMB 05036) and TB *Mycobacterium tuberculosis* (RCMB 010094-8).

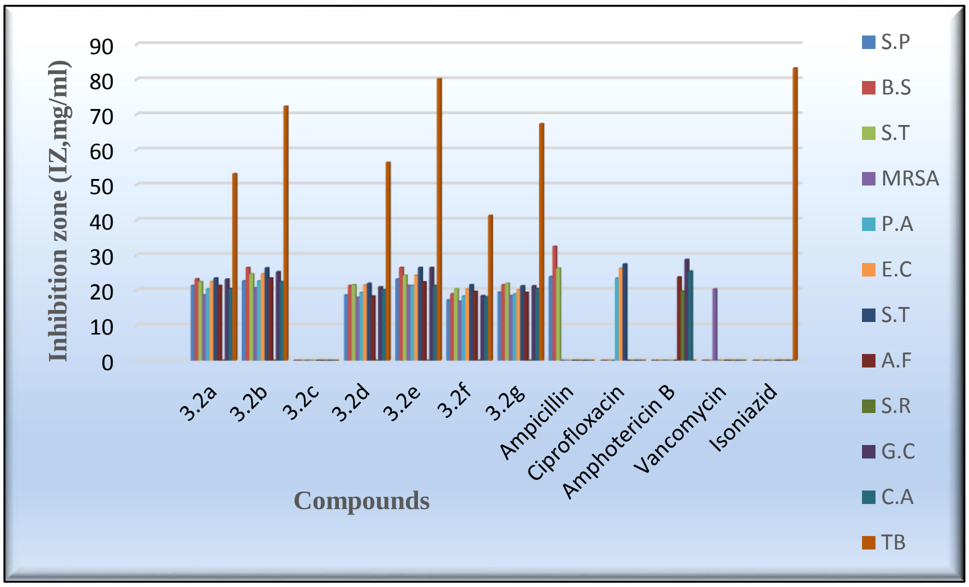


Figure 3.3: Evaluation of Inhibition zone values (IZ) of synthesized derivatives.

Table 3.3: Antimicrobial activity of synthetic compounds (MIC, µg/ml).

Compounds	Minimal inhibitory concentration (MIC, µg/ml)											TB
	Gram-positive				Gram-negative			Fungi				
	S.P	B.S	S.A	MRSA	P.A	E.C	S.T	A.F	S.R	G.C	C.A	
3.2a	1.95	0.98	1.95	7.81	3.9	1.95	0.98	1.95	NA	0.98	3.9	31.2
3.2b	0.98	0.49	0.49	3.9	0.98	0.49	0.49	0.98	NA	0.49	0.98	15.6
3.2d	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	31.2
3.2e	0.98	0.49	0.98	1.95	1.95	0.98	0.49	0.98	NA	0.49	1.95	3.9
3.2f	15.63	3.9	3.9	15.63	7.81	3.9	0.98	3.9	NA	7.81	7.81	62.5
3.2g	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	7.8
Ampicillin	0.98	0.24	0.49	-	-	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	0.99	0.59	0.56	-	-	-	-	-
Isoniazid	-	-	-	-	-	-	-	-	-	-	-	1.95
Amphotericin B	-	-	-	-	-	-	-	0.98	3.9	0.49	0.49	-
Vancomycin	-	-	-	3.9	-	-	-	-	-	-	-	-

Minimum inhibitory concentration (MIC, mg/mL), results are mean values from at least three experiments (triplicate, mean ± SE, with standard errors range 0.1-0.7). (NA) means no activity.

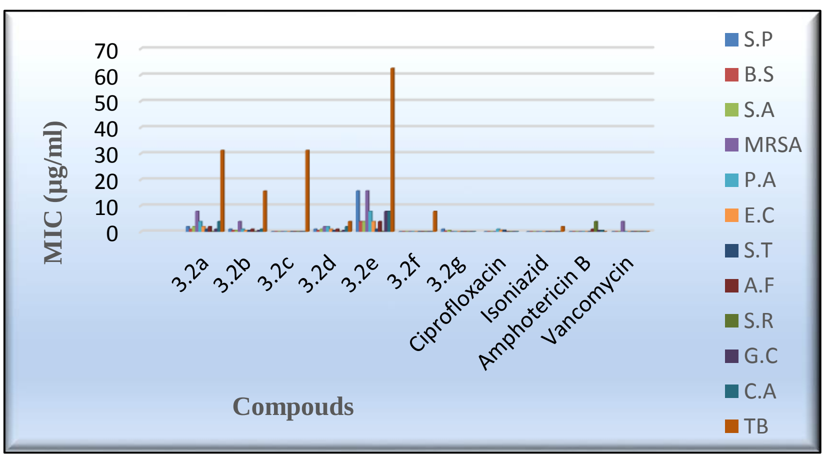


Figure 3.4: Evaluation of minimum inhibitory concentration values (MIC) of compounds.

3.3.2.2 Cytotoxic Screening

The *in vitro* cytotoxic activity of the synthesized derivatives **3.2(a-g)** was tested against four human carcinoma cell lines including human colon carcinoma (HCT-116), human hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7), and adenocarcinoma human alveolar basal epithelial cell (A-549) cell lines. The assay was performed by MTT assay[121,122] Doxorubicin was used as reference drug. The inhibitory activities (IC_{50}) are given in **Table 3.4** and **Figure 3.5**. Compounds **3.2b** and **3.2(d-g)** showed good cell growth inhibitory activity against HCT-116 cell line (IC_{50}= 1.08-1.48 µg/ml). The IC_{50} values of test compounds against HepG-2 and A-549 cells revealed that compound **3.2e** had comparable activity respect to the reference drug. By comparing the inhibitory activity of compounds with that of doxorubicin against MCF-7 cell line, most compounds were less active than the doxorubicin. Further research might lead to improvement in the inhibitory activity especially for compound **3.2e** against HCT-116 cell line.

Compounds	**IC_{50} (µg/mL)**			
	HCT-116	**MCF-7**	**HepG-2**	**A-549**
3.2a	6.09	16.1	18.2	11.6
3.2b	1.41	5.56	5.26	4.79
3.2c	7.48	9.4	4.38	2.81
3.2d	1.33	2.9	3.07	3.01
3.2e	1.08	2.42	2.04	1.39
3.2f	1.13	3.53	29.2	9.23
3.2g	1.48	12.2	2.58	2.63
Doxorubicin	0.88	1.02	1.19	0.91

Table 3.4: Cytotoxicity of chromene derivatives against four different cancer cell lines.

Cytotoxicity activity results from at least three experiments (triplicate, mean ±SE, with standard errors range 0.02- 0.7). Cytotoxic effects of compounds on colon, breast, liver, and epithelial cell lines following exposure to different concentrations of compounds, and cell viability was assessed

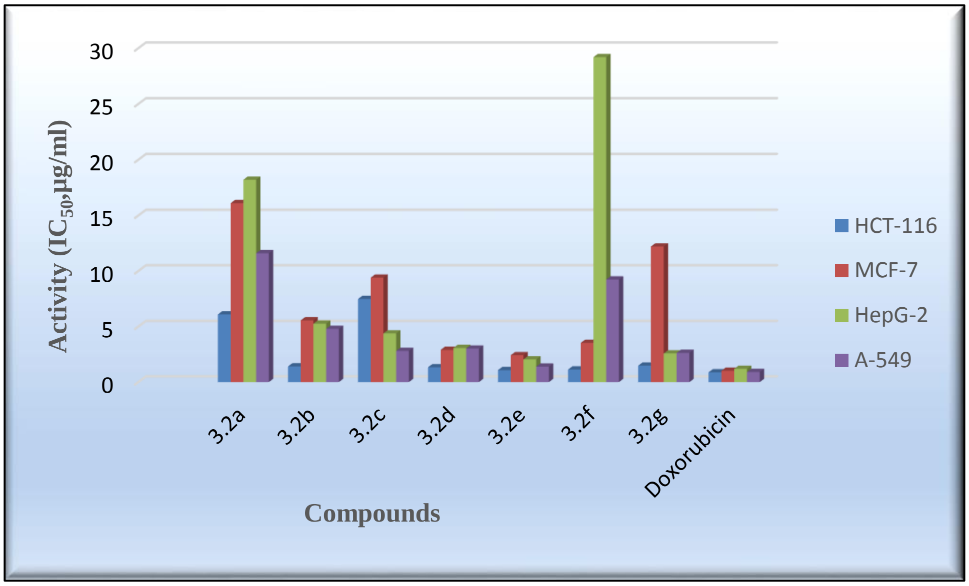


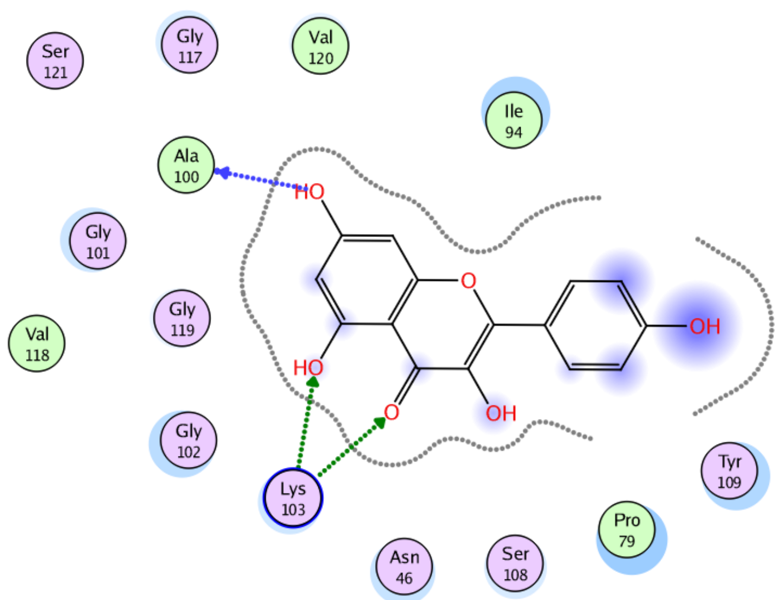
Figure 3.5: Evaluation of cytotoxic activity of target compounds compared to reference drug.

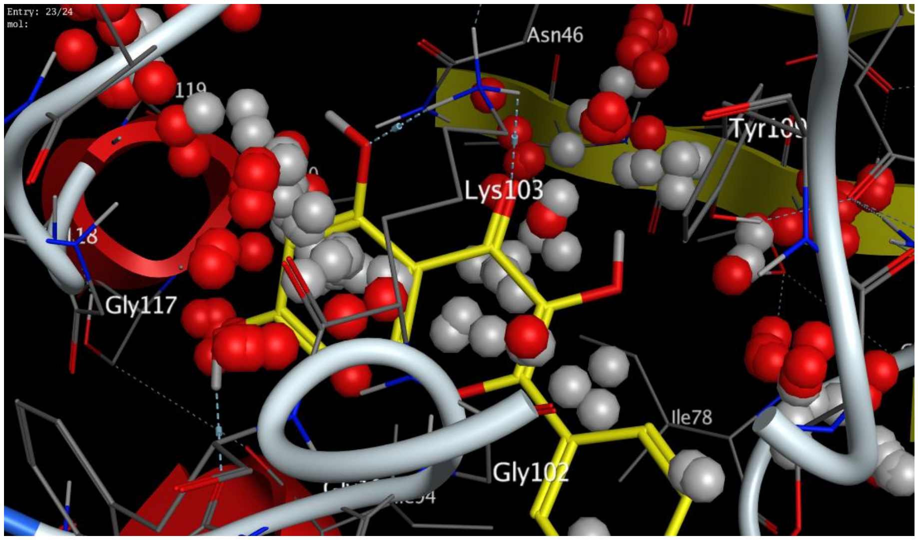
2.3.3 Computational studies

3.3.3.1 Docking studies

Docking study was performed for tested compounds, **3.2a**, **3.2b**, **3.2d** and **3.2e** in order to investigate the possible interactions with Gyrase B (GyrB43) from *E. coli* (PDB code: 4PRV; resolution 2.00 Å) by dock module implemented in MOE software. It has been reported as a good target for studying antibacterial activity.[155]

Figure 3.6 present best docking poses for new synthesized chromenes inside gyrase B binding pocket. The bound kaempferol displayed interaction including hydrogen bonding interactions with Ala 100 (2.29 Å), Lys 103 (1.94, 2.16 A) for hydroxyl group linked to C5 and carbonyl group. The results of the docking experiments are presented in **Table 3.5**. The binding map of the compounds in the pocket is explained through different fragments, 4-C=O, 5-OH, 4`-OH, 8-NH_2, 3-CH, 9-CN and phenyl. These fragments form stable hydrogen bonding interactions with a panel of corresponding pocket residues: Ala 100, Glu 50, Asp 73, Lys 103, Gly 77, Gly 102 and Pro 79.

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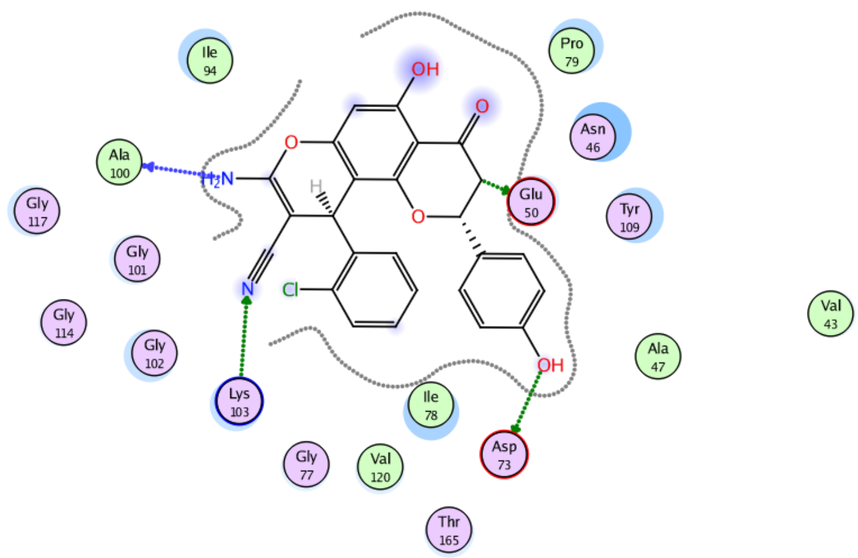
Lys103
Tyr109
Gly117
Ile78
Gly102

Kaempferol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Pro 79
Asn 46
Ala 100
Gly 117
Glu 50
Tyr 109
Gly 101
Val 43
Gly 114
Gly 102
Ala 47
Lys 103
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Gly 77
Val 120
Thr 165
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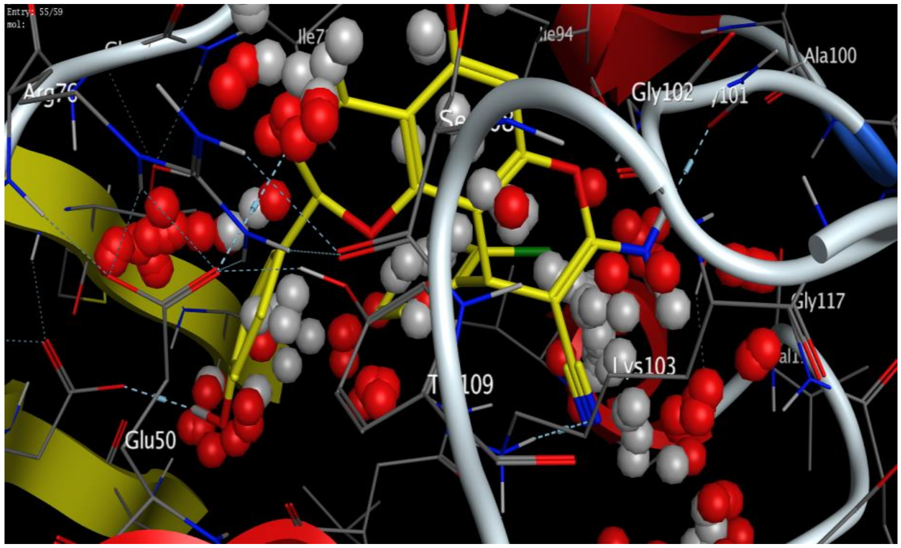
O
O
H2N
H
N
Cl
OH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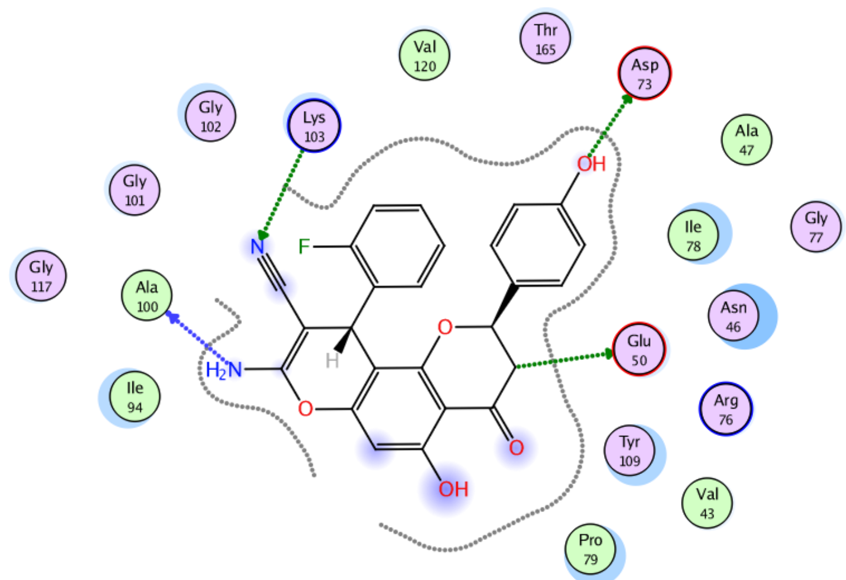
Lys103
Glu50

3.2a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Thr 165
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Gly 102
Lys 103
Ala 47
Gly 101
OH
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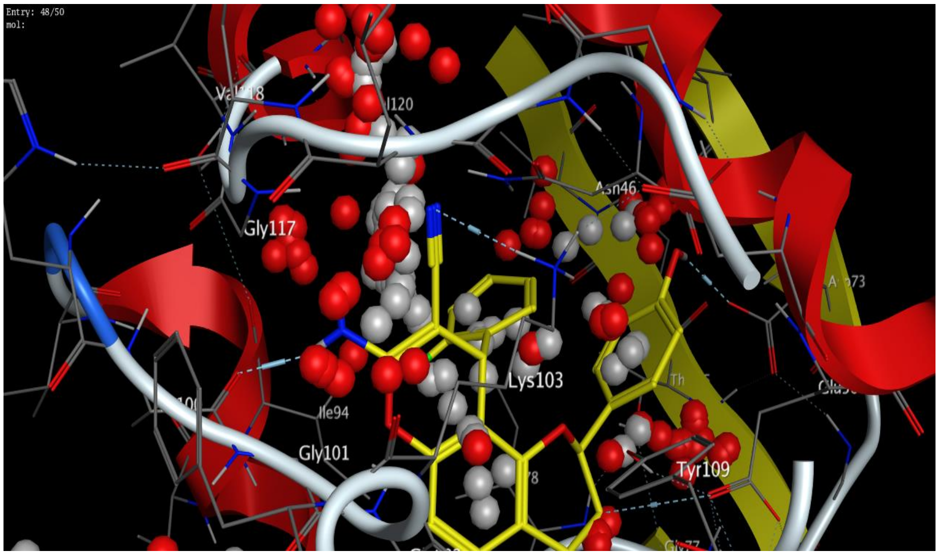
Glu 50
H
H_2N
Ile 94
O
Arg 76
O
Tyr 109
OH
Val 43
Pro 79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mol:
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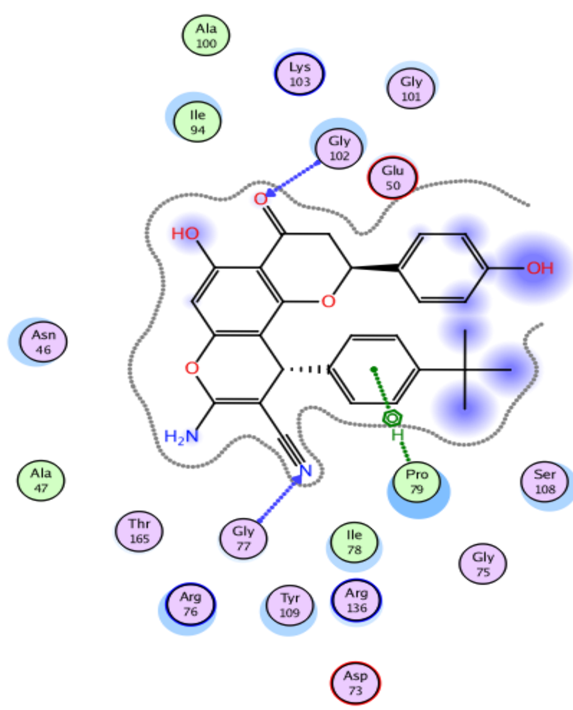
Asn46
Lys103
Ile94
Gly101
Tyr109

3.2b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Ile 94
Gly 102
Glu 50
HO
O
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OH
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H
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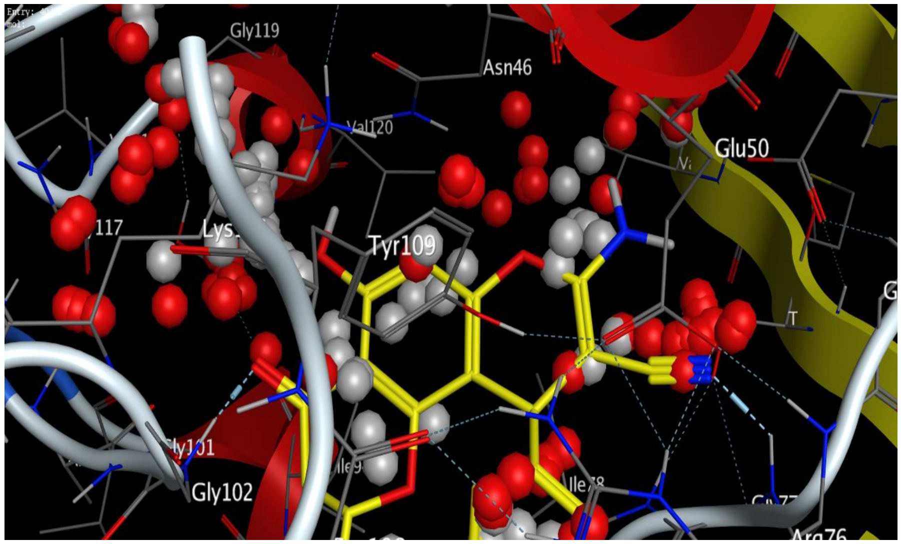
Ser 108
Thr 165
Gly 77
Ile 78
Gly 75
Arg 76
Tyr 109
Arg 136
Asp 73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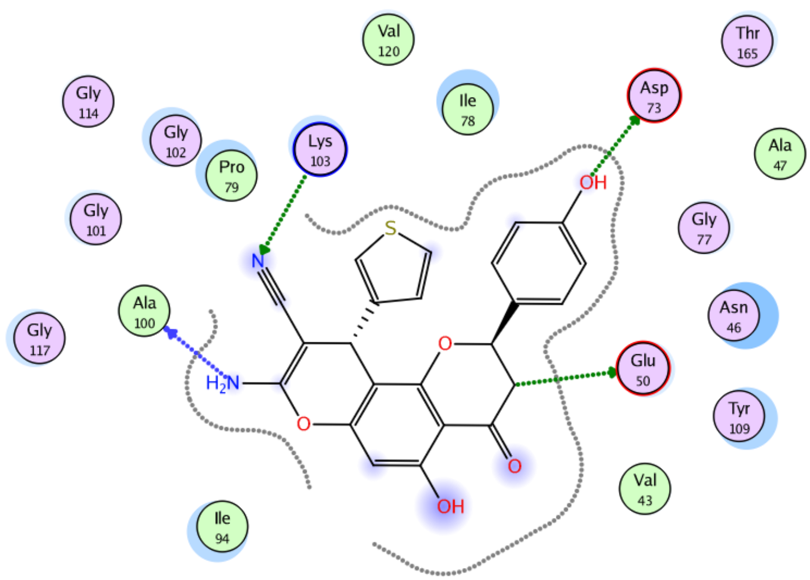
3.2d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Asp 73
Gly 102
Lys 103
Pro 79
Ala 47
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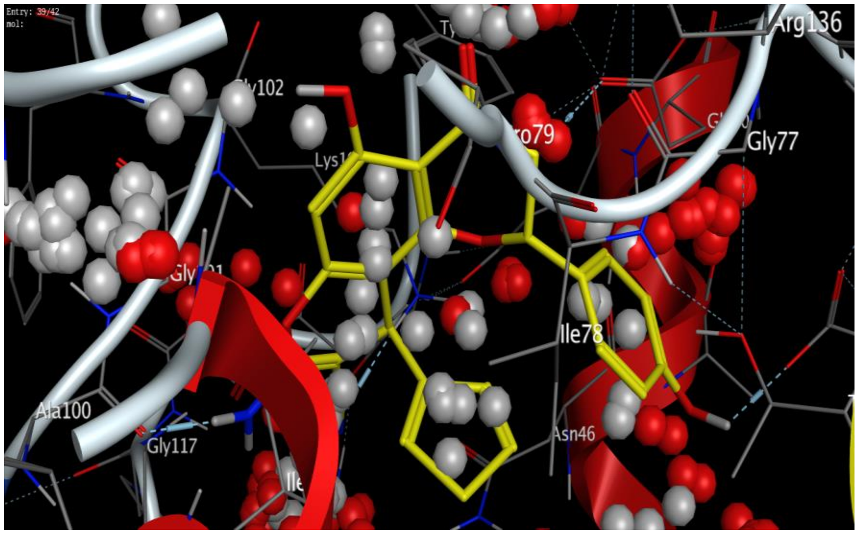
S
N
OH
O
H2N



3.2e

Figure 3.6: 2D and 3D binding interaction of target compounds; naringenin, **3.2a**, **3.2b** **3.2d** and **3.2e** inside DNA gyrase enzyme subunit B

Table 3.5: Description of the docking data of the selected target compounds, **3.2e**, **3.2b** **3.2d** and **3.2a**

Compound No.		3.2a	3.2b	3.2d	3.2e	Kaempferol
Amino acid (Distance Å)	Ala 100	$-NH_2$ (2.1)	$-NH_2$ (1.8)	-	$-NH_2$ (1.7)	-OH (2.2)
	Glu 50	-CH (3.2)	-CH (3.2)		CH (3.3)	-
	Asp 73	-OH (1.8)	-OH (1.8)	-	OH (1.8)	-
	Lys 103	-CN (2.2)	-CN (2.5)-	-	-CN (2.4)	-OH, C=O (1.94, 2.16)
	Gly 77	-	-	-C=O (1.7)	-	-
	Gly 102	-	-	-CN (1.79)	-	-
	Pro 79	-	-	Phenyl (2.7)	-	-
	Asn 46	-	-	-	-	-
Interaction type		H-bonding	H-bonding	H-bonding (Aromatic)	H-bonding	H-bonding
ΔG (kcal/mol)		-14.10	-15.65	-15.35	-16.00	-12.02

The data reported in the table are extracted from MOE program showing the corresponding amino acids residues in enzyme pocket, corresponding fragments of ligands, interaction distances, types of interaction, and their binding energy to some selected drugs compared to reference drug.

3.4 Conclusions

The present study describes the synthesis of new 8-amino-10-phenyl-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-diydro-2*H*,10*H*-pyrano [2,3-*f*] chromene derivatives **3.2(a-g)** and evaluation of their antimicrobial activity and cytotoxic effects. These target compounds were synthesized by condensation of benzaldehyde derivatives and malononitrile in an ethanolic piperidine (Knoevenagel condensation) followed by the electrophilic substitution step of 4`5,7-trihydroxy-flavanone (Michael addition adduct). The structures of the synthesized compounds were established from spectroscopic data. The results of antibacterial assay revealed that the compounds **3.2a, 3.2b** and **3.2(d-g)** exhibited the highest inhibitory activity against P.A and *E. coli* by IZ range 18.3 to 24.6 mm and MIC 0.49 to 3.9 µg/ml compared to reference drugs. Compounds **3.2a**, **3.2b** and **3.2e** were found to be more effective against S.T by IZ 23.4-26.4 mm. The antibacterial activity of most compounds was found to be comparably active to reference drugs against Gram-positive bacteria. Moreover, the cytotoxic activity was also evaluated against four different human carcinoma cell lines. Most of compounds have good inhibitory activity against HCT-116 cell line (IC_{50}= 1.08-1.48 µg/ml). Finally, compound **3.2c** showed no any antimicrobial activity against all the tested bacteria, fungi and TB. The molecular modeling results showed binding interaction of new synthesized derivatives in the active site of Gyrase B.

3.5 Experimental Section

3.5.1 Materials and Instrumentation

Chemicals and solvents were purchased from Sigma-Aldrich (Canada) and Alfa Aesar, and were used as received. Compound **3.1** was purchased from Sigma-Aldrich (Canada). Melting points were determined in open capillaries using an electrothermal apparatus and are uncorrected. The progress of the reactions was monitored using thin layer chromatography (TLC) on Merck silica gel 60 F254 plates. Infrared (IR) spectra were recorded using Bruker Alpha FT-IR Spectrometer as pressed KBr pellets. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 300 MHz and at 75 MHz, respectively, on a Bruker Avance Spectrometer in DMSO-d_6 with tetramethylsilane as internal standard. The high vacuum for overnight and heat gun were used to removed residual water from the compounds. Elemental analyses for C, H and N were performed using an Exeter Analytical, Inc. CE-440 Elemental Analyzer.

3.5.2 Biological Studies

3.5.2.1 *Antimicrobial Screening*

The microorganism inoculums were uniformly spread using sterile cotton swabs on a sterile Petri dish malt extract agar (for fungi) and nutrient agar (for bacteria). One hundred cubic millimeters of each sample was added to each well (10-mm-diameter holes were cut in the agar gel, 20 mm apart from one another). The systems were incubated for 24–48 hr. at 37^{o}C (for bacteria) and at 28^{o}C (for fungi).

After incubation, microorganism growth was observed. Inhibition zones of the bacterial and fungal growth were measured in millimeters. Tests were performed in triplicate. [113,114]

3.5.2.2 *Cytotoxic Screening*

Human colon carcinoma (HCT-116), human hepatocellular carcinoma (HEPG-2), adenocarcinomic human alveolar basal epithelial cell (A-549), and human breast adenocarcinoma (MCF-7) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 μg/mL gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. Potential cytotoxicity of the compounds was evaluated on tumor cells using the method of Gangadevi and Muthumary.[130] The cells were grown as monolayers in growth RPMI-1640. The monolayers of 104 cells adhered at the bottom of the wells in a 96-well microliter plate incubated for 24 h at 37ºC in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M pH 7.2) and simultaneously the cells were treated with 100 µL from different dilutions of tested sample in fresh maintenance medium and incubated at 37ºC. A control of untreated cells was made in the absence of tested sample. Positive controls containing doxorubicin were also tested as a reference drug for comparison. Six wells were used for each concentration of the test sample. Every 24-hour observation under the inverted microscope was made.

The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and the absorbance read at 590 nm using a microplate reader (SunRise, TECAN, Inc, USA) through mixing. [121,131] The absorbance values from untreated cells were considered as 100% proliferation. The number of viable cells was determined using microplate reader as previously mentioned and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration was plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50 % inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

3.5.3 Molecular modeling

The newly synthesized compounds were docked into the crystal structure of *E. coli* topoisomerase II DNA gyrase B (PDB code 1KZN). The MOE software[124] was used for all docking calculations. The MOE tools package was employed to generate the docking input files and to analyze the docking results. All non-polar hydrogens, clorobiocin, and crystal water molecules were removed prior to the calculations and the protonation of the enzyme was carried out and was energy minimized. In each case, 100 docked structures were generated using genetic algorithm searches. The 3D structures of new synthesized compounds were drawn in MOE and the protonation of ligands were carried out.

The energy of compounds was minimized up to 0.05 gradient using GBVI/WSA dG force field. These data were saved in database as input file MOE. Heavy atom comparison root means square deviations (RMSD values) were calculated and initial ligand binding modes were plotted. Protein-ligand interaction plots were generated using MOE 2012.10. Quantum mechanical calculations and surface molecular orbitals were generated by simulation module in MOE software.

3.5.4 Synthesis

3.5.4.1 General procedure for the synthesis of synthesis of 8-amino-10-phenyl-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihydro-2H,10H-pyrano[2,3-f] chromene-9-carbonitrile derivatives 3.2(a-g).

Aryl aldehyde (2.3 mmol) was added to a solution containing compound **3.1** (2.3 mmol) in 5 mL of ethanol and malononitrile or ethyl cyanoacetate (2.3 mmol) with few drops of piperidine. The reaction mixture was stirred under reflux. After completion of the reaction (monitored by TLC), the mixture was kept at room temperature and the formed solid product was collected by filtration and washed with ethanol and hexane to yield **3.2(a-g).**

3.5.4.2 8-amino-10-(2-chlorophenyl)-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihydr o -2H,10H-pyrano[2,3-f] chromene-9-carbonitrile (3.2a).

White solid (0.75g, 89.51 %), m.p. 262°C; IR (KBr) cm^{-1}: 3489 (OH), 3360 (NH_2), 2178 (CN), 1634 (C=O); ^{1}H NMR (DMSO, 300 MHz) δ 12.3 (s, 1H, C5-OH), 9.63 (s, 1H, C4`-OH), 7.39 (d, *J* = 7.5 Hz, 1H, H3``), 7.33 (d, *J* = 8.5 Hz, 2H, diastereomeric H2` & H6`), 7.32 (d, *J* = 8.5 Hz, 2H, diastereomeric H2` & H6`), 7.29-7.18 (m, 2H, H4`` & H5``), 7.15-7.04 (m, 3H, overlapping H6`` & NH_2), 6.80 (d, *J* = 8.5 Hz, 2H, H3` & H5`), 6.23 (s, 1H, H6), 5.55 (dd, *J* = 12.8, 2.8 Hz, 1H, diastereomeric H2), 5.53 (dd, *J* = 12.8, 2.8 Hz, 1H, diastereomeric H2), 5.08 (s, 1H, H10), 3.40 (dd, *J* = 17.1, 13.0 Hz, 1H, diastereomeric trans H3ax), 3.41 (dd, *J* = 17.1, 13.0 Hz, 1H, diastereomeric trans H3ax), 2.73 (dd, *J* = 17.1, 2.7 Hz, 1H, diastereomeric cis H3eq), 2.70 (dd, *J* = 17.1, 2.7 Hz, 1H, diastereomeric cis H3eq); ^{13}C NMR (DMSO, 75 MHz) δ 198.9 (C=O), 162.1 (C-5), 160.5 (C-13), 160.2 (C-8), 158.8 (C-4), 156.6 (C-12), 142.6 (diastereomeric C-1``), 142.5 (diastereomeric C-1``), 132.9 (C-2``), 132.8 (C-1`), 131.3 (diastereomeric C-4``), 131.2 (diastereomeric C-4``), 130.4 (C-3``), 129.4 (C-2` & C-6`), 129.2 (C-5``), 128.4 (C-6``), 120.3 (CN), 116.1 (C-3` & C-5`), 105.9 (C-14), 103.8 (C-11), 95.7 (diastereomeric C-6), 95.6 (diastereomeric C-6), 79.8 (diastereomeric C-2), 79.7 (diastereomeric C-2), 56.7 (diastereomeric C-9), 56.6 (diastereomeric C-9), 43.0 (diastereomeric C-3), 42.7 (diastereomeric C-3), 34.0 (C-10); Anal. Calcd for $C_{25}H_{17}N_2O_5Cl$: C, 65.15; H, 3.72; N, 6.08. Found: C, 65.34; H, 3.63; N, 6.02

3.5.4.3 8-amino-10-(2-fluorophenyl)-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihydro -2H,10H-pyrano[2,3-f] chromene-9-carbonitrile (3.2b).

Yellow solid (0.52g, 71.51 %), m.p. 290°C; IR (KBr) cm^{-1}: 3497 (OH), 3403, 3192 (NH_2), 2198 (CN), 1647 (C=O); ^{1}H NMR (DMSO, 300 MHz) δ 12.4 (s, 1H, C5-OH), 9.63 (s, 1H, C4`-OH), 7.32 (d, $J$ = 8.5 Hz, 1H, diastereomeric H2` & H6`), 7.31 (d, $J$ = 8.5 Hz, 2H, diastereomeric H2` & H6`), 7.29-7.20 (m, 1H, H4``), 7.20-7.11 (m, 3H, H3``, H5`` & H6``), 7.09 (s, NH_2), 6.80 (d, J = 8.5 Hz, 2H, H3`, H5`), 6.22 (s, 1H, H6), 5.55 (dd, J = 12.2, 2.7 Hz, 1H, diastereomeric H2), 5.52 (dd, J = 12.6, 2.5 Hz, 1H, diastereomeric H2), 4.82 (s, 1H, H10), 3.51-3.37 (m, 1H, diastereomeric trans H3ax), 2.73 (dd, J = 17.1, 2.7 Hz, 1H, diastereomeric cis H3eq), 2.70 (dd, J = 17.1, 2.7 Hz, 1H, diastereomeric cis H3eq); ^{13}C NMR (DMSO, 75 MHz) δ 198.0 (C=O), 162.6 (C-2``), 162.2 (C-5), 159.5 (C-13), 159.3 (C-8), 157.9 (C-4`), 155.7 (C-12), 129.9 (C-1``), 128.5 (C2` & C-6`), 128.3 (C-1`), 128.2 (C-4``), 124.5 (C-5``), 132.5 (C-6``), 119.7 (CN), 115.5 (diastereomeric C-3``), 115.2 (C-3`), 115.1 (diastereomeric C-3``), 107.9 (diastereomeric C-14), 104.7 (diastereomeric C-14), 102.8 (diastereomeric C-11), 100.1 (diastereomeric C-11), 94.8 (diastereomeric C-6), 94.8 (diastereomeric C-6), 79.0 (diastereomeric C-2), 78.8 (diastereomeric C-2), 56.0 (diastereomeric C-9), 55.7 (diastereomeric C-9), 42.2 (diastereomeric C-3), 41.8 (diastereomeric C-3), 30.3 (diastereomeric C-10), 30.3 (diastereomeric C-10); Anal. Calcd for $C_{25}H_{17}N_2O_5F$: C, 67.57; H, 3.86; N, 6.30. Found: C, 67.24; H, 3.98; N, 6.09

3.5.4.4 8-amino-10-(3-bromophenyl)-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihydro -2H,10H-pyrano[2,3-f] chromene-9-carbonitrile (3.2c).

White solid (0.72g, 77.42 %), m.p. 182°C; IR (KBr) cm^{-1}: 3634(OH), 3424 (NH_2), 2207 (CN), 1653 (C=O); 1H NMR (DMSO, 300 MHz) δ 12.4 (s, 1H, C5-OH), 9.63 (s, 1H, C4`-

OH), 7.45-7.37 (m, 2H, H2` & H6`), 7.36-7.23 (m, 3H, H2``, H4`` & H5``), 7.21-7.10 (m, 3H, H6`` & NH_2), 6.80 (d, J = 8.5 Hz, 2H, H3` & H5`), 6.24 (s, 1H, H6), 5.56 (dd, J = 12.5, 2.8 Hz, 1H, diastereomeric H2), 5.53 (dd, J = 13.1, 2.8 Hz, 1H, diastereomeric H2), 4.62 (s, 1H, diastereomeric H10), 4.61 (s, 1H, diastereomeric H10), 3.42 (dd, J = 17.2, 13.5 Hz, 1H, diastereomeric trans H3ax), 3.41 (dd, J = 17.6, 13.3 Hz, 1H, diastereomeric trans H3ax), 2.75 (dd, J = 17.4, 3.0 Hz, 1H, diastereomeric cis H3eq), 2.71 (dd, J = 17.1, 2.7 Hz, 1H, diastereomeric cis H3eq); 13C NMR (DMSO, 75 MHz) δ 198.9 (C=O), 162.9 (C-5), 160.4 (C-13), 160.3 (C-8), 158.8 (C-4'), 156.2 (C-12), 148.5 (C-1''), 131.7 (C-2''), 130.6 (C-5''), 129.4 (C-2' & C-6`), 129.2 (C-1'), 128.5 (C-4''), 127.2 (C-6''), 122.5 (C-3''), 120.7 (CN), 116.1 (C-3` & C-5`), 105.7 (C-14), 104.3 (C-11), 96.0 (C6-H), 79.8 (C-2), 57.7 (C-9), 43.0 (diastereomeric C-3), 42.9 (diastereomeric C-3), 36.2 (C-10);Anal. Calcd for $C_{25}H_{17}N_2O_5Br$: C, 59.42; H, 3.39; N, 5.54. Found: C, 59.74; H, 3.28; N, 5.31.

3.5.4.5 8-amino-10-(4-tert-butylphenyl)-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihyd -ro-2H,10H-pyrano[2,3-f] chromene-9-carbonitrile (3.2d).

Yellowish white solid (0.74g, 84.36 %), m.p. 264°C; IR (KBr) cm^{-1}: 3488 (OH), 3381 (NH_2), 2961- 2869 (CH), 2185 (CN), 1643 (C=O); *Diastereomeric ratio: 1:1;* ^{1}H NMR (DMSO, 300 MHz) δ 11.9 (s, 1H, C5-OH), 9.62 (s, 1H, C4`-OH), 7.30 (d, *J* = 8.3 Hz, 2H, H3``), 7.03 (br s, 2H, $NH_2$), 6.94 (d, *J* = 8.2 Hz, 2H, H2``), 6.91 (d, *J* = 8.0 Hz, 2H, H2` & H6`), 6.74 (d, *J* = 8.1 Hz, 2H, H3` & H5`), 6.21 (s, 1H, H6), 5.07 (dd, *J* = 12.8, 2.5 Hz, 1H, H2), 4.46 (s, 1H, H10), 3.34 (dd, J = 17.1, 13.2 Hz, 1H, trans H3ax), 2.75 (dd, J = 17.1, 2.7 Hz, 1H, cis H3eq); 1.33 (s, 9H, 3CH_3); ^{13}C NMR (DMSO, 75 MHz) δ 198.7 (C=O), 161.9 (C-5), 160.2 (C-13), 160.0 (C-8), 158.7 (C-4\`), 156.3 (C-12), 149.7 (C-4\`\`), 142.9 (C-1\`\`), 129.4 (C2\` & C-6\`), 127.7 (C-1\`), 127.4 (C-2\`\`), 126.0 (C-3\`\`), 120.9 (CN), 115.9 (C-3\` & C-5\`), 106.2 (C-14), 104.4 (C-11), 96.8 (C-6), 79.9 (C-2), 58.0 (C-9), 41.7 (C-3), 36.2 (C-10), 35.0 (t-Bu quaternary carbon), 32.0 (CH_3); Anal. Calcd for $C_{29}H_{26}N_2O_5$: C, 72.18; H, 5.43; N, 5.81. Found: C, 71.99; H, 5.20; N, 5.73.

3.5.4.6 8-amino-10-(thiophene)-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihydro-2H-10H-pyrano[2,3-f] chromene-9-carbonitrile (3.2e).

White solid (0.68g, 88.31 %), m.p. 293°C; IR (KBr) cm^{-1}: 3501 (OH), 3402, 3205 (NH_2), 2206 (CN), 1653 (C=O); ^{1}H NMR (DMSO, 300 MHz) δ 12.5 (s, 1H, C5-OH), 9.63 (s, 1H, C4\`-OH), 7.46-7.37 (m, 1H, H4\`\`), 7.32 (d, $J$ = 8.3 Hz, 2H, H2\` & H6\`), 7.20-7.14 (m, 1H, H2\`\`), 7.11 (br s, 2H, $NH_2$), 6.92-6.85 (m, 1H, H5\`\`), 6.81 (d, $J$ = 8.3 Hz, 2H, H3\` & H5\`), 6.21 (s, 1H, H6), 5.59-5.43 (m, 1H, H2), 4.73 (s, 1H, diastereomeric H10), 4.71 (s, 1H, diastereomeric H10), 3.40 (dd, $J$ = 17.3, 13.2 Hz, 1H, trans H3ax), 2.83-2.68 (m, 1H, cis H3eq); $^{13}$C NMR (DMSO, 75 MHz) δ 198.9 (C=O), 161.9 (C-8), 160.7 (C-13), 160.2 (C-5), 158.8 (C-4\`), 156.3 (C-12), 146.2 (C-1\`\`), 129.4 (C-2\` & C-6\`), 129.1 (C-1\`), 127.5 (diastereomeric C-5\`\`), 127.4 (diastereomeric C-5\`\`), 127.3 (C-4\`\`), 121.8 (diastereomeric

C-2``), 121.7 (diastereomeric C-2``), 121.0 (CN), 116.1 (C-3` & C-5`), 106.7 (diastereomeric C-14), 106.6 (diastereomeric C-14), 105.3 (diastereomeric C-11), 105.2 (diastereomeric C-11), 96.0 (diastereomeric C-6), 95.9 (diastereomeric C-6), 79.8 (diastereomeric C-2), 79.7 (diastereomeric C-2), 57.6 (diastereomeric C-9), 57.5 (diastereomeric C-9), 43.1 (diastereomeric C-3), 42.8 (diastereomeric C-3), 31.5 (C-10); Anal. Calcd for $C_{23}H_{16}N_2O_5S$: C, 63.88; H, 3.73; N, 6.48. Found: C, 63.64; H, 3.60; N, 6.16.

3.5.4.7 8-amino-10-(4-fluorophenyl)-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihydro-2H,10H-pyrano[2,3-f] chromene-9-carbonitrile (3.2f).

Yellow solid (0.54g, 68.35 %), m.p. 276°C; IR (KBr) cm^{-1}: 3494 (OH), 3400, 3197 (NH_2), 2193 (CN), 1649 (C=O); ^{1}H NMR (DMSO, 300 MHz) δ 12.4 (s, 1H, C5-OH), 9.63 (s, 1H, C4`-OH), 7.32 (d, *J* = 8.5 Hz, 1H, diastereomeric H2` & H6`), 7.31 (d, *J* = 8.5 Hz, 2H, diastereomeric H2` & H6`), 7.23-7.11 (m, 4H, H2``, H6``, H5`` & H3``), 7.10 (s, NH_2), 6.80 (d, *J* = 8.5 Hz, 2H, H3` & H5`), 6.23 (s, 1H, H6), 5.54 (dd, *J* = 12.8, 3.0 Hz, 1H, diastereomeric H2), 5.53 (dd, *J* = 13.1, 3.0 Hz, 1H, diastereomeric H2), 4.61 (s, 1H, diastereomeric H10), 4.59 (s, 1H, diastereomeric H10), 3.41 (dd, *J* = 17.4, 13.2 Hz, 1H, diastereomeric trans H3ax), 2.74 (dd, *J* = 17.1, 2.9 Hz, 1H, diastereomeric cis H3eq), 2.71 (dd, *J* = 17.3, 2.9 Hz, 1H, diastereomeric cis H3eq); ^{13}C NMR (75 MHz, DMSO-*d6*) δ 198.82 (C=O), 163.37 (C-4``), 161.93 (C-5), 159.92 (C-13), 159.36 (C-8), 157.56 (C-4`), 154.95 (C-12), 129.91 (C-1``), 131.39.81 (C-2` & C-6`), 130.07 (C-1`),), 129.72 (C-2`` & 6``), 128.47 (C-3`` & C-5``), 120.74 (CN), 115.99 (diastereomeric C-3` & C-5`),116.85 (diastereomeric C-3` & C-5`) 105.64 (C-14), 104.97 (C-11), 95.88 (diastereomeric C-6), 95.76 (diastereomeric C-6), 79.78 (diastereomeric C-2), 79.67 (diastereomeric C-2), 53.58 (C-9), 42.75 (C-3), 36.09 (C-10Anal. Calcd for $C_{25}H_{17}N_2O_5F$: C, 67.57; H, 3.86; N, 6.30. Found: C, 67.27; H, 3.74; N, 6.12

3.5.4.8 Ethyl-8-amino-10-(3-nitrophenyl)-5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-3,4-dihydro-2H,10H-pyrano[2,3-f] chromene-9-carboxlate (3.2g).

White solid (0.71g, 74.58 %), m.p. 230°C; IR (KBr) cm^{-1}: 3477 (OH), 3411, 3294 (NH_2), 2914- 2880 (CH), 1686 (C=O); 1528, 1349 (NO); ^{1}H NMR (DMSO, 300 MHz) δ 11.9 (s, 1H, C5-OH), 9.70 (s, 1H, diastereomeric C4`-OH), 9.62 (s, 1H, diastereomeric C4`-OH), 8.08-7.98 (m, 1H, H4``), 7.88 (s, 1H, diastereomeric H2``), 7.79 (br s, 2H, NH_2), 7.62-7.35 (m, 2H, H5`` & H6``), 7.11 (d, *J* = 8.7 Hz, 2H, diastereomeric H2` & H6`), 7.06 (d, *J* = 8.7 Hz, 2H, diastereomeric H2` & H6`), 6.79 (d, *J* = 8.4 Hz, 2H, diastereomeric H3 & H5`), 6.72 (d, *J* = 8.6 Hz, 2H, diastereomeric H3` & H5`), 6.27 (s, 1H, diastereomeric H6), 6.26 (s, 1H, diastereomeric H6), 5.64 (dd, *J* = 12.5, 2.9 Hz, 1H, diastereomeric H2), 5.07 (dd, *J* = 13.6, 2.5 Hz, 1H, diastereomeric H2), 4.90 (s, 1H, diastereomeric H10), 4.83 (s, 1H, diastereomeric H10), 4.03-3.81 (m, 2H, O**CH_2**CH_3) 3.45 (dd, *J* = 17.2, 13.4 Hz, 1H, diastereomeric trans H3ax), 3.11 (dd, *J* = 17.4, 12.9 Hz, 1H, diastereomeric trans H3ax), 2.72 (dd, J = 17.6, 3.1 Hz, 1H, diastereomeric cis H3eq), 2.66 (dd, J = 17.2, 2.7 Hz, 1H, diastereomeric cis H3eq); 0.98 (t, 3H, $OCH_2\mathbf{CH_3}$); ^{13}C NMR (DMSO, 75 MHz) δ 198.7 (diastereomeric C=O), 198.3 (diastereomeric C=O), 168.7 (Ester C=O), 162.1 (diastereomeric C-5), 162.0 (diastereomeric C-5),160.8 (diastereomeric C-13), 160.7 (diastereomeric C-13), 160.0 (diastereomeric C-8), 159.9 (diastereomeric C-8), 158.9 (diastereomeric C-4\`), 158.5 (diastereomeric C-4\`), 156.1 (C-12), 149.6 (diastereomeric C-3\`\`), 149.6 (diastereomeric C-3\`\`), 148.0 (C-1\`\`), 135.2 (diastereomeric C-6\`\`), 135.0 (diastereomeric C-6\`\`), 130.5 (diastereomeric C-5\`\`), 130.4 (diastereomeric C-5\`\`), 129.5 (diastereomeric C-2\` & C-6\`), 128.5 (diastereomeric C-2\` & C-6\`), 128.4 (C-1\`), 123.4 (diastereomeric C-4\`\`), 123.3 (diastereomeric C-4\`\`), 122.0 (diastereomeric C-2\`\`), 121.9 (diastereomeric C-2\`\`), 116.0 (diastereomeric C-3\` & C-5\`), 115.9 (diastereomeric C-3\`& C-5\`), 106.6 (diastereomeric C-14), 106.7 (diastereomeric C-14), 104.3 (C-11), 96.7 (diastereomeric C-6), 96.6 (diastereomeric C-6), 80.2 (diastereomeric C-2), 80.0 (diastereomeric C-2), 76.8 (diastereomeric C-9), 76.6 (diastereomeric C-9), 59.8 ($O\mathbf{C}H_2CH_3$), 43.4 (diastereomeric C-3), 43.0 (diastereomeric C-3), 35.3 (diastereomeric C-10), 35.3 (diastereomeric C-10), 15.0 ($OCH_2\mathbf{C}H_3$); Anal. Calcd for $C_{27}H_{22}N_2O_9$: C, 62.55; H, 4.28; N, 5.40. Found: C, 62.19; H, 3.99; N, 5.32.

Chapter Four: Synthesis, Antimicrobial, Cytotoxicity and Docking Studies of a New Family of Chromene Azo Dyes.

4.1 Synopsis

A variety of novel derivatives of 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4*H*-benzo[*h*]chromene-3-carbonitrile **4.2(a-j)** have been prepared *via* three component reaction of 1-naphthalenol-4-[(4-ethoxyphenyl) azo] **4.1** with benzaldehyde derivatives and malononitrile. The structures were confirmed on the basis of their spectral data and elemental analysis. Their antimicrobial activity was investigated and tested against four human pathogen Gram-positive and Gram-negative bacteria: *Streptococcus pneumoniae*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and four fungi including *Aspergillus fumigatus*, *Syncephalastrum racemosum, Geotricum candidum*, and *Candida albicans*, using agar well diffusion method and minimum inhibitory concentrations were reported. Some newly prepared compounds exhibited antimicrobial activities against gram-negative and fungi species compared to reference drugs. Compounds **4.2a, 4.2g, and 4.2h** were found to be more effective against *Syncephalastrum racemosm* (RCMB 05922) by IZ range 20.1 to 21.4 mm and MIC 1.95 to 3.9 µg/ml compared to reference drugs, while in the case of the Gram-negative bacteria, the compounds **4.2b** and **4.2h** showed the highest inhibitory activity against *E. coli* by IZ range 20.4 to 21.2 mm and MIC = 3.9 µg/ml compared to reference drugs. The bathochromic shift of compound **4.2a** was shown in solution when acidified.

Moreover, the cytotoxic activity was also evaluated against three different human cell lines including human colon carcinoma (HCT-116), human hepatocellular carcinoma (HepG-2), human breast adenocarcinoma (MCF-7), and adenocarcinoma human alveolar basal epithelial cell (A-549) cell lines. Some of tested compounds have good IC_{50} ranging from 4.35 to 5.54 μg/mL against HCT-116 and MCF-7 cell line. The molecular modeling results showed binding interaction of synthesized compounds **3.2b**, **3.2h** in the active site of enzyme.

4.2 Introduction

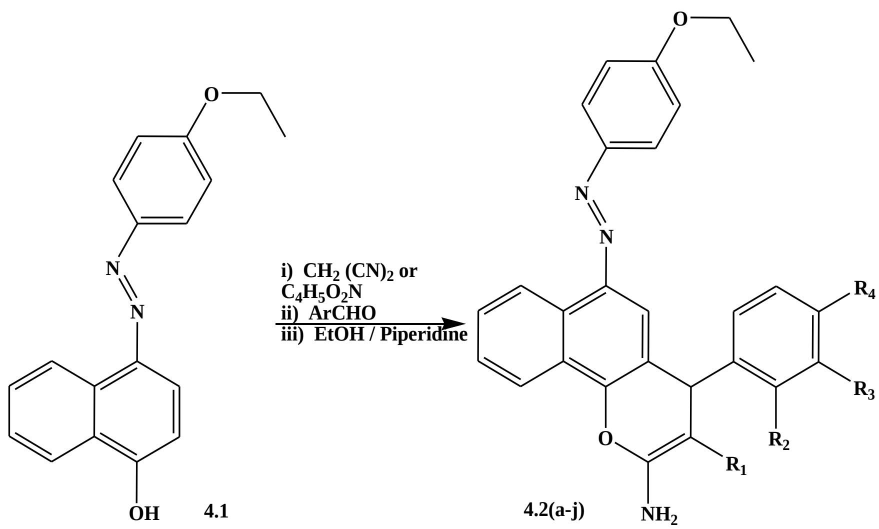
4*H*-Chromenes are one class of the most biologically active heterocyclic compounds. The pharmacological and biochemical properties of simple 4H-chromenes depend upon pattern of substitution. In recent years,4*H*-chromene and its derivatives have attracted intense interest due to their diverse biological properties.[4] The 4*H*-chromenes have long been recognized to possess antimicrobial and anticarcinogenic activates. These properties of 4*H*-chromenes are interesting to warrant the synthesis of new compounds featuring different heterocyclic rings fused to the chromene moiety in order to obtain more active compounds. On the other hand, azo dyes are characterized with presence of azo moiety (-N=N-) in their structure. They have found broad applications in analytical chemistry, pharmaceutical, cosmetic, food and dyeing. Additionally, azo dyes have various biological activities such as antibacterial, antifungal and anti-HIV properties.[85] Biological activity of azo compounds result from specific reduction of azo bound.

In this reaction the azo bond is cleaved to release aromatic amine from the azo dye.[156] This process can decrease or increase any toxic effect of the dyes. [157,158] The present study is focused on the possibility of synthesizing novel 4*H*-chromene compounds fused to azo dye moieties and studying *in vitro* antimicrobial activity and cytotoxic effects for these new derivatives.

4.3 Results and Dissection

4.3.1 Synthesis and Characterization

In our attempts to synthesize chromene derivatives, 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4*H*-benzo[*h*]chromene-3-carbonitrile derivatives were obtained in good yield by three component condensation of 1-naphthalenol-4-[(4-ethoxyphenyl) azo] (Fat Brown B) **4.1** with benzaldehyde derivatives and malononitrile or ethyl cyanoacetate under reflux using piperidine in ethanol, **(Table 4.1)**. Knoevenagel condensation followed by Michael addition adducts is the common method for this synthesis[113], as shown in the following **Scheme 4.1**

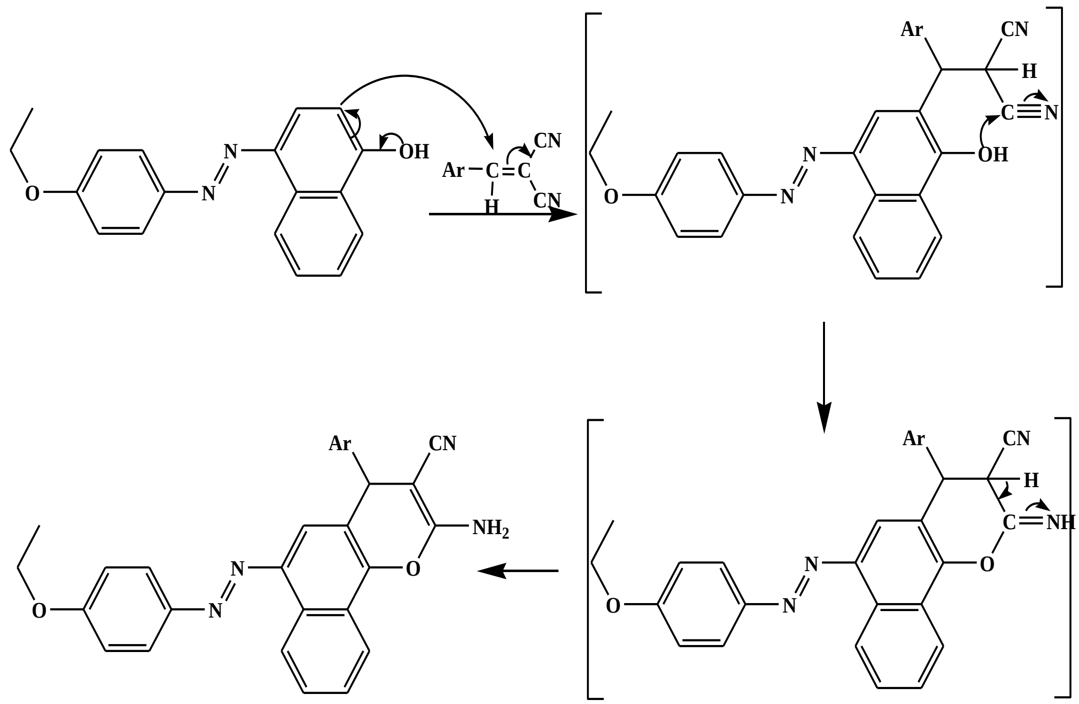


Scheme 4.1: Synthesis of 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4*H*-benzo[*h*]chrom -ene derivatives **4.2(a-j)**

Compound No.	R1	R2	R3	R4	Yield%
4.2a	CN	F	H	H	62.05
4.2b	CN	Cl	H	H	74.92
4.2c	CN	Br	H	H	58.29
4.2d	CN	H	Br	H	60.57
4.2e	CN	H	Cl	H	67.43
4.2f	CN	H	H	F	55.58
4.2g	CN	H	NO_2	H	65.99
4.2h	$COOCH_3$	Br	H	H	58.43
4.2i	$COOCH_3$	Cl	H	H	50.78
4.2j	$COOCH_3$	H	Br	H	78.26

Table 4.1: The yields of new synthesized derivatives **4.2(a-j)**

The first step of this multicomponent process begins with Knoevenagel condensation and then followed by Michael addition adducts[85], as shown in the following **Scheme 4.2**.



Scheme 4.2: General synthesis and mechanistic pathway of chromene molecules **4.2(a-j)**

The structures of new chromene compounds **4.2(a-j**) were confirmed with the aid of spectroscopic data. FT-IR spectroscopy showed characteristic absorption bands between 2187 and 2210 cm^{-1} for the CN groups while the NH_2 stretches were in the range of 3336-3470 cm^{-1}. The ^1H NMR spectra of chromene compounds **4.2(a-j**) were obtained in DMSO-d_6. As anticipated, the methyl protons appeared as a triplet set at 1.34-1.37 ppm, while the H4 pyran showed signals at 5.07-5.49 ppm and the amine protons resonated at 6.71-7.11 ppm. The singlet at 4.11-4.19 ppm corresponds to the methylene group.

The rest of the aromatic protons resonate further downfield in the range of 6.62-8.97 ppm**.** **Figure 4.1** shows one example of the proton NMR for compound **4.2c**.

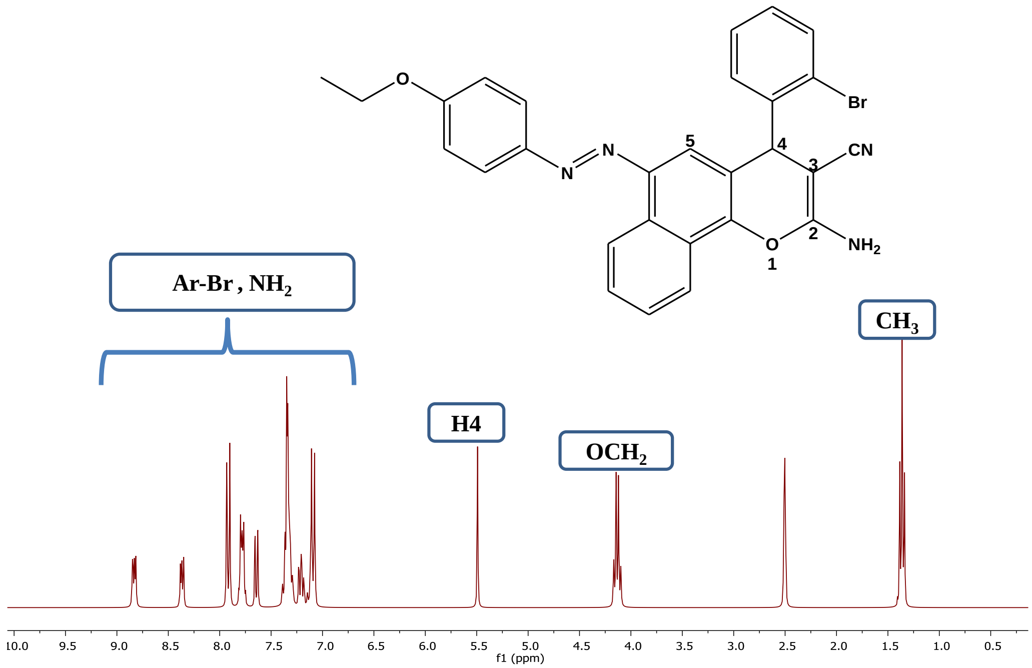


Figure 4.1: ^{1}H NMR of Compound **4.2c**

The^{13}C NMR spectra of the synthesized compounds showed that the carbon atoms attached to the methyl protons resonate at 15.40-14.48 ppm, while the methylene carbons resonate at 64.58-64.14 ppm. The signal at 41.71-31.52 ppm corresponds to the C-4 of the pyran ring, while the quaternary carbon C-2 that is attached to the amine group appeared in the range of 58.49-55.67 ppm.

The CN carbon resonated further downfield at 118.92-117.69 ppm and the aromatic CH carbons showed signals between 144.06 and 111.75 ppm. **Figure 4.2** shows ^{13}C NMR spectrum of **4.2c** as example.

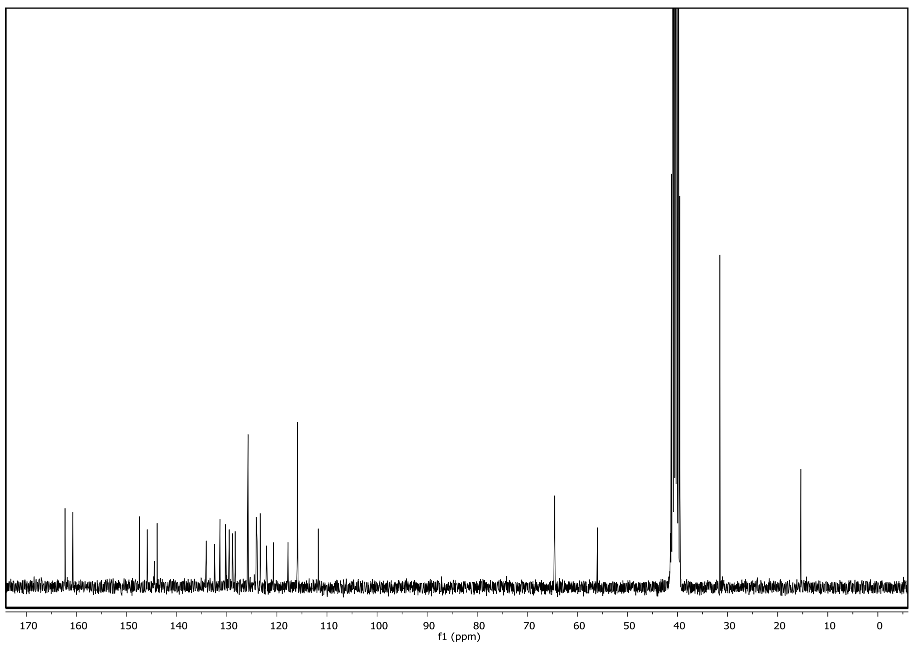


Figure 4.2: ^{13}C NMR of Compound **4.2c**

The absorption spectra of the new synthesized compounds **4.2(a-j)** in DMF showed two bands around 393 and 591 nm with almost similar width as shown in **Figure 4.3**.

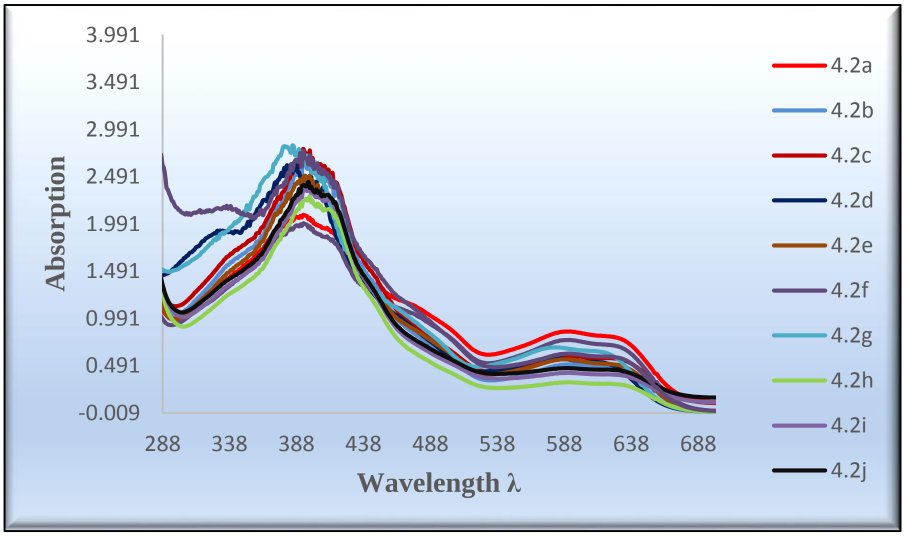


Figure 4.3: UV-vis absorption spectra of compounds **4.2(a-j)** (10µM) in DMF

Addition of an acidic solution to these compounds caused a bathochromic shift in the absorption maxima. For example, after addition of acid to **4.2a** two absorption bands observed at 524 and 408 nm as shown in **Figure 4.4**.

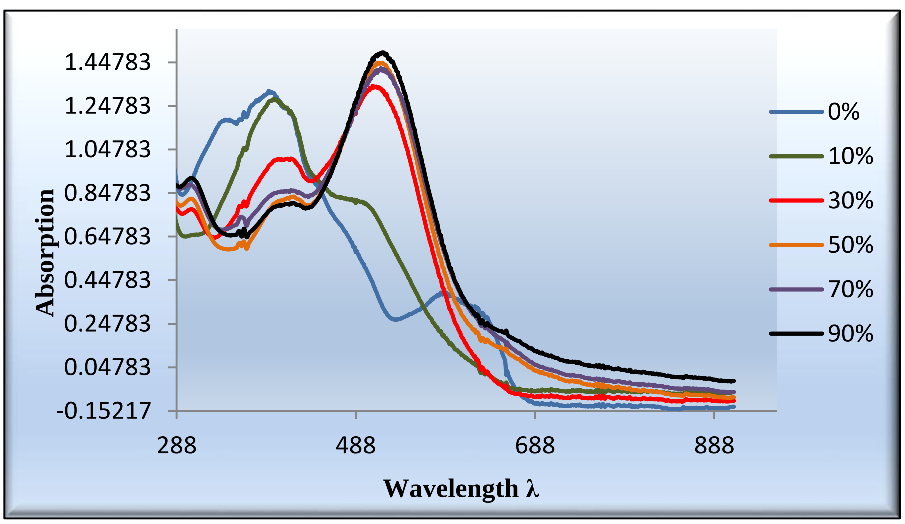


Figure 4.4: wavelength of **4.2a** in the presence of increasing [H^+] (HCl) in a DMF

In the same time the colour of DMF solution of compound **4.2a** was changed when exposed to H^+, due to the formation of the azonium form of the azo group allowing for the visual identification of aqueous acid. Compound **4.2a** for example, changed from yellow/green in DMF to orange as the concentration of H^+ increased and as concentration increased further the colour changed from orange to dark pink (**Figure 4.5**)

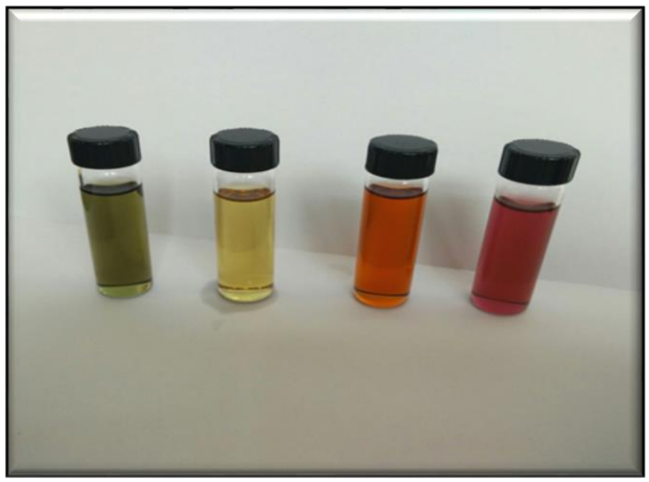


Figure 4.5: Colour change of **4.2a** in the presence of increasing $[H^+]$ (HCl) in a DMF.

4.3.2 Biological Screening

4.3.2.1 Antimicrobial screening

The synthesized compounds **4.2(a-j)** in this chapter were screened for their antibacterial, and antifungal, activities via agar diffusion well method.[117] The inhibition zones and minimum inhibitory concentrations (MIC) were determined by serial dilution method.[118]

The activity of the synthesized compounds was tested against two Gram-positive bacteria including *Streptococcus pneumoniae* (RCMB 010010), *Bacillus subtilis* (RCMB 010067), two Gram-negative bacteria including *Pseudomonas aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052), and four fungi including *Aspergillus fumigatus* (RCMB 02568), *Syncephalastrum racemosum* (RCMB 05922*), Geotricum candidum* (RCMB 05097), and *Candida albicans* (RCMB 05036). Ampicillin, gentamicin and amphotericin B were used as control drugs. [119,120] The observed inhibition zone (IZ) and minimum inhibitory concentrations (MIC) of the compounds and the reference drugs are given in **Table 4.2**, **4.3** and presented in **Figure 4.6**, **4.7**. Among the synthesized compounds **4.2a, 4.2g, 4.2h and 4.2i** were found to be more effective against *Syncephalastrum racemosm* (RCMB 05922) by IZ range 20.1 to 21.4 mm and MIC 1.95 to 3.9 µg/mL compared to reference drugs. The values of inhibition zone (IZ) and minimum inhibitory concentrations (MIC) of the target compounds against A.F and G.C revealed that most of the synthesized compounds were found to be comparable active to the reference drugs. In the case of the Gram-negative bacteria, the highest inhibitory activity against *E. coli* was showed by IZ range 20.4 to 21.2 mm and MIC = 3.9 µg/ml for compounds **4.2b** and **4.2h** compared to reference drugs. The synthesized compounds were moderately or slightly active against the S.P and B.S, while all the compounds did not show any antimicrobial activity against P.A and C.A. Generally, the investigation of antimicrobial activity of the novel derivatives showed greater than the reference drugs against *E. coli* and S.R and mild or slightly activity towards gram-positive bacteria.

Table 4.2: Antimicrobial activity of synthetic compounds (IZ, diameter (mm)) (1 g/mL).

Compounds	Inhibition Zone Diameter (mm)							
	Gram-positive		Gram-negative		Fungi			
	S. P	B. S	P. A	E.C	A. F	S.R	G.C	C.A
4.2a	20.3	20.9	NA	15.4	18.6	20.1	20.6	NA
4.2b	18.4	12.2	NA	20.4	20.9	19.2	22.8	NA
4.2c	17.4	20.2	NA	17.3	16.2	15.3	17.1	NA
4.2d	19.6	20.0	NA	15.9	18.2	17.3	18.9	NA
4.2e	20.3	23.4	NA	19.4	18.6	15.3	19.2	NA
4.2f	20.3	21.4	NA	16.9	17.6	18.4	20.6	NA
4.2g	18.9	20.3	NA	18.9	16.3	20.3	21.4	NA
4.2h	21.4	23.4	NA	21.2	23.3	21.4	25.2	NA
4.2i	19.6	21.4	NA	19.3	18.2	20.3	20.8	NA
4.2j	16.0	18.3	NA	13.0	10.6	18.7	23.4	NA
Ampicillin	23.8	32.4	-	-	-	-	-	-
Gentamicin	-	-	17.3	19.9	-	-	-	-
Amphotericin B	-	-	-	-	23.7	19.7	28.7	25.4

Mean zone of inhibition in mm from at least three experiments; (triplicate, mean ± SE, with standard errors range 0.01- 0.8). (NA) means no activity. S.P (*Streptococcus pneumoniae*), B.S (*Bacillus subtilis*), P.A (*Pseudomonas aeruginosa*), E.C (*Escherichia coli*), S.T (*Salmonella typhimurium*), A.F *Aspergillus fumigatus* (RCMB 02568), G.C *Geotricum candidum* (RCMB 05097), S.R *Syncephalastrum racemosum* (RCMB 05922), and C.A *Candida albicans* (RCMB 05036).

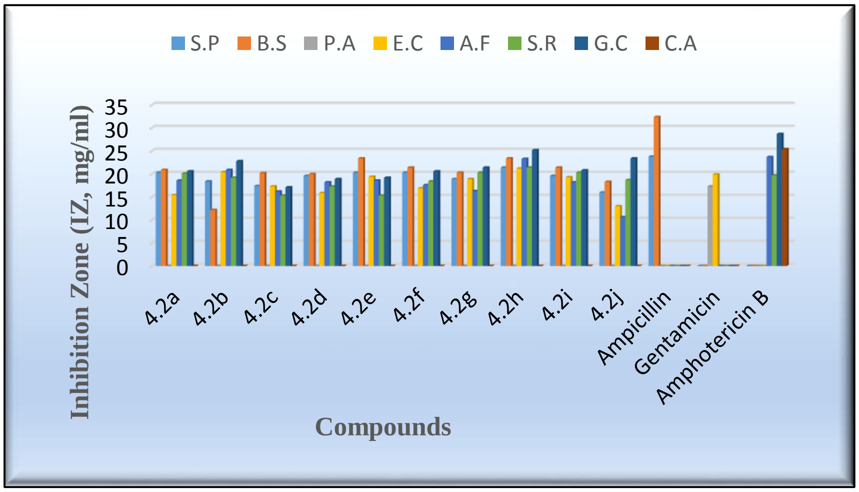


Figure 4.6: Evaluation of Inhibition zone values (IZ) of synthesized derivatives.

Compounds	Minimal inhibitory concentration (MIC, µg/ml)							
	Gram-positive		Gram-negative		Fungi			
	S.P	B.S	P.A	E.C	A.F	S.R	G.C	C.A
4.2b	7.81	1.95	NA	3.9	1.95	3.9	0.98	NA
4.2g	3.9	3.9	NA	1.95	3.25	3.9	1.95	NA
4.2h	1.95	0.98	NA	3.9	0.98	1.95	0.49	NA
Ampicillin	0.98	0.24	-	-	-	-	-	-
Gentamicin	-	-	15.63	3.9	-	-	-	-
Amphotericin B	-	-	-	-	0.98	3.9	0.49	0.49

Table 4.3: Antimicrobial activity of synthetic compounds (MIC, µg/mL).

Minimum inhibitory concentration (MIC, mg/mL), results are mean values from at least three experiments (triplicate, mean ± SE, with standard errors range 0.1-0.7). (NA) means no activity.

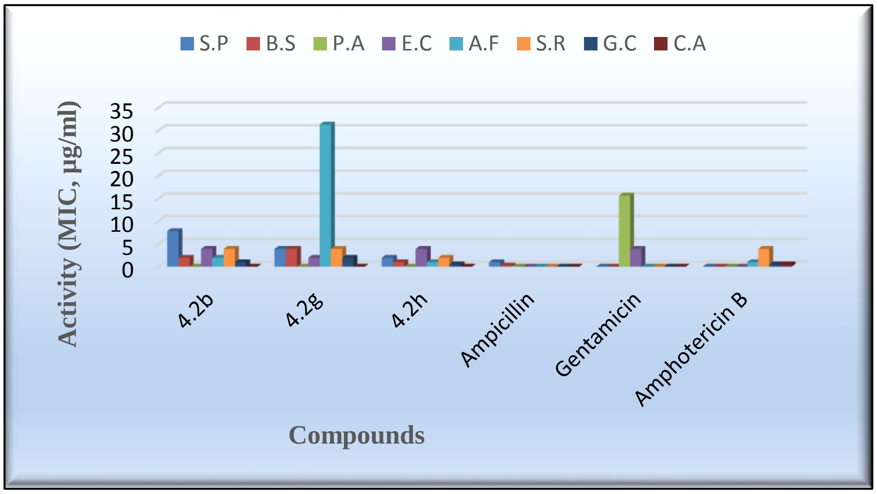


Figure 4.7: Evaluation of minimum inhibitory concentration values (MIC). of compounds.

4.3.2.2 Cytotoxic screening

The *in vitro* cytotoxic activity was performed by MTT assay[121,122] against three human carcinoma cell lines: Human colon carcinoma (HCT-116) human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG-2) cell lines. Doxorubicin was used as a positive control, since it has high cytotoxic activity. The inhibitory effects of synthesized compounds **4.2(a-j)** on the growth of the three cell lines are shown in **Table 4.4** and **Figure 4.8**. All compounds showed comparable or slight cytotoxicity to the reference drug. The new synthesized derivatives **4.2e**, **4.2h** and **4.2j** exhibited good IC_{50} ranging from 4.35 to 5.54 µg/mL against of HCT-116 and MCF-7 cell lines, while all compounds showed less activity in case of HepG-2 cell line.

Table 4.4: Cytotoxicity of chromene derivatives against three different cancer cell lines.

Compounds	**IC_{50} (µg/mL)**		
	HCT-116	**MCF-7**	**HepG-2**
4.2a	24.1	21.9	11.6
4.2b	19.2	16.2	11.3
4.2c	12.2	21.1	7.28
4.2d	15.3	9.16	7.64
4.2e	5.54	19.7	9.99
4.2f	24.6	22.5	14.3
4.2g	9.01	7.14	12.0
4.2h	10.9	5.5	9.19
4.2i	23.0	11.5	8.76
4.2j	4.35	4.72	11.6
Doxorubicin	0.88	1.02	1.19

Cytotoxicity activity results from at least three experiments (triplicate, mean ±SE, with standard errors range 0.02- 0.7). Cytotoxic effects of compounds on colon, breast, liver, and epithelial cell lines following exposure to different concentrations of compounds, and cell viability was assessed

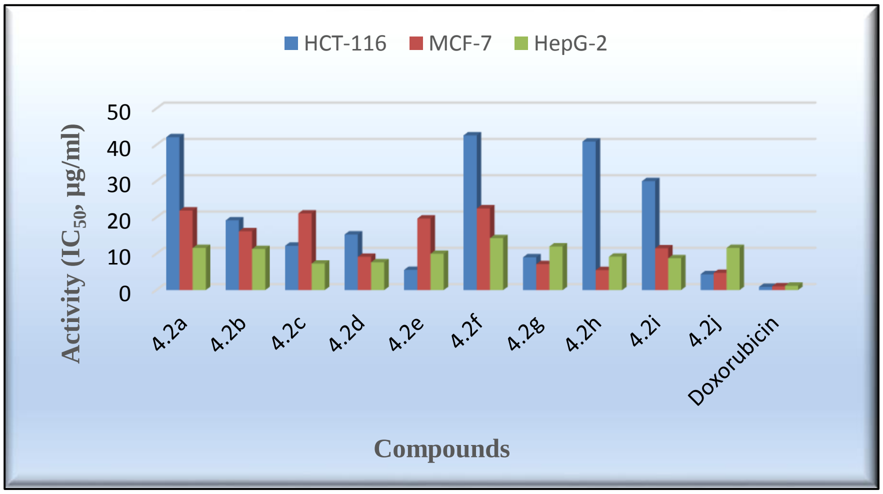


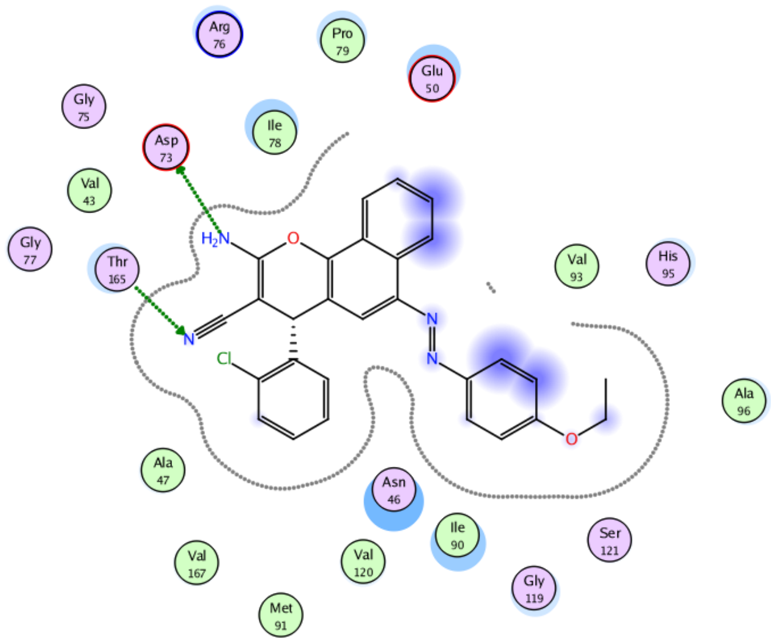
Figure 4.8: Evaluation of cytotoxic activity of target compounds compared to reference drug.

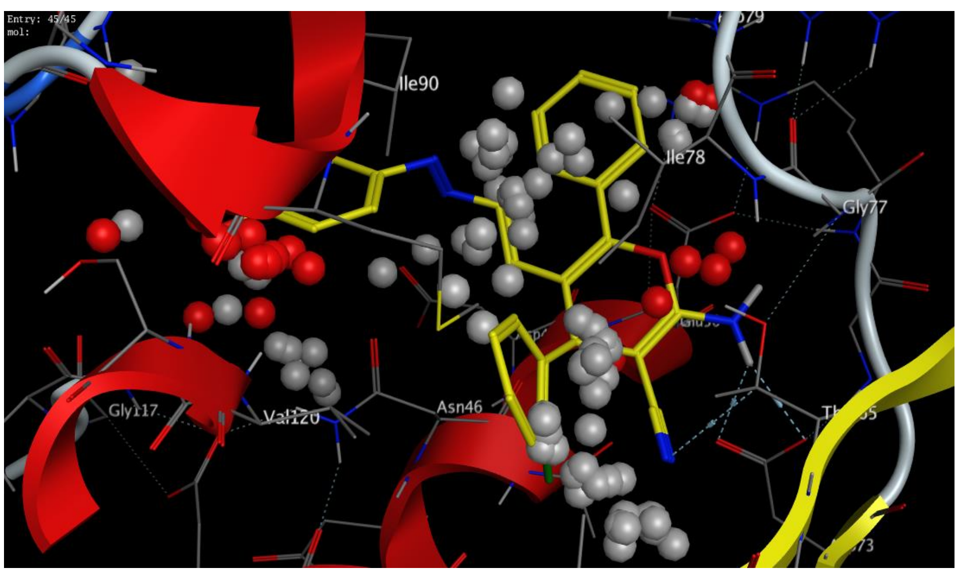
4.3.3 Computational studies

4.3.3.1 Docking studies

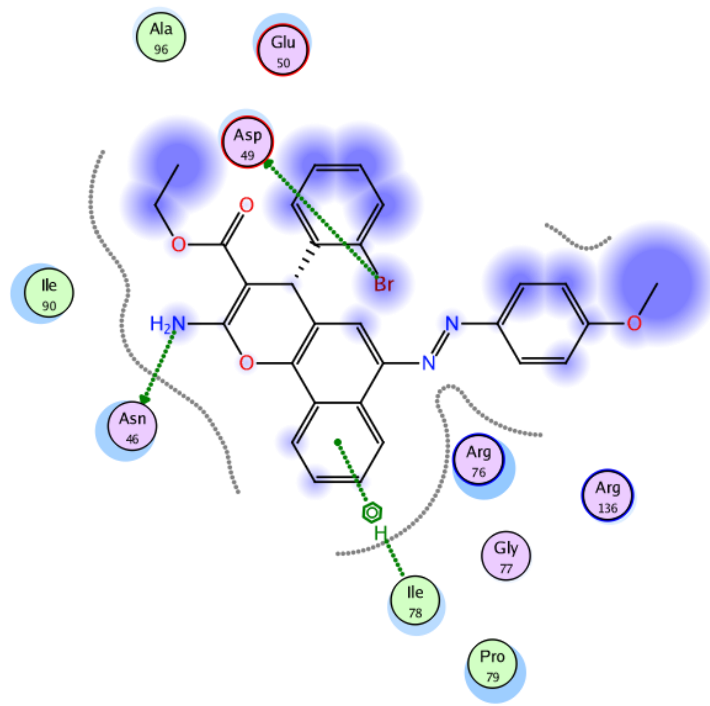
The molecular docking calculations of the synthesized derivatives **4.2b** and **4.2h** were performed to gain insight into the plausible mechanism of antibacterial activity of the target compounds. Molecular modeling studies using the DNA gyrase B subunit were performed, as it has been reported as a good target for studying inhibitory activity against these bacteria.[123]. *E. coli* topoisomerase II DNA gyrase B (responsible for the super-coiling activity of DNA in bacteria) (PDB code: 1KZN; resolution 2.30 Å) was used by docking module implemented in MOE software. [124,125] The least energy binding mode of the compounds has been studied. **Figure 4.9** presents the best docking poses for all investigated chromene derivatives inside topoisomerase II DNA gyrase B binding pocket.

The bound fat brown B displayed interaction including hydrogen bonding interaction with Asp 73 (2.45 Å)





4.2b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Glu
50
Asp
49
O
O
Ile
90
H2N
O
Br
N
N
O
Asn
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Arg
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Arg
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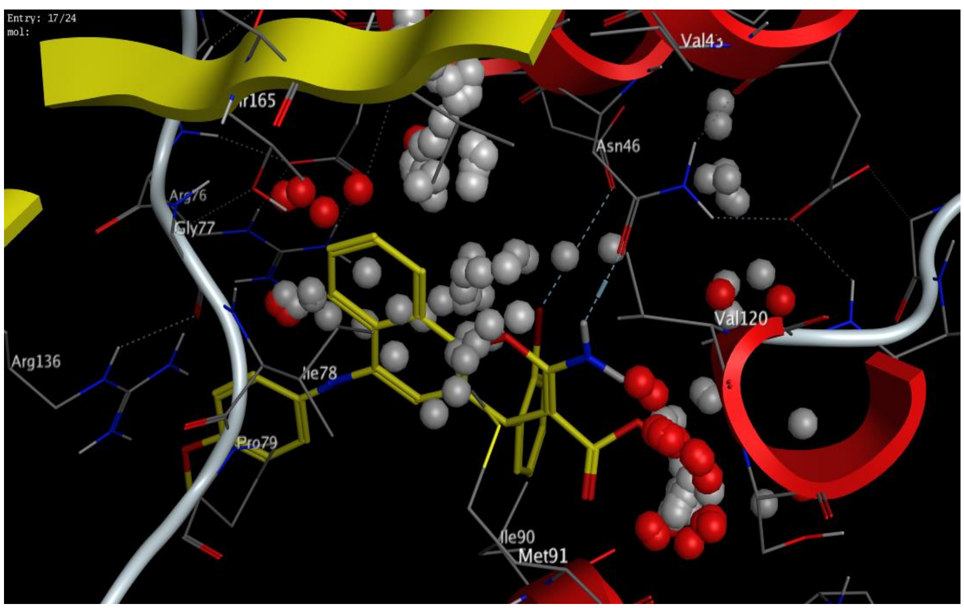
H
Gly
77
Ile
78
Pro
79


Entry: 17/24
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Asn46
Arg76
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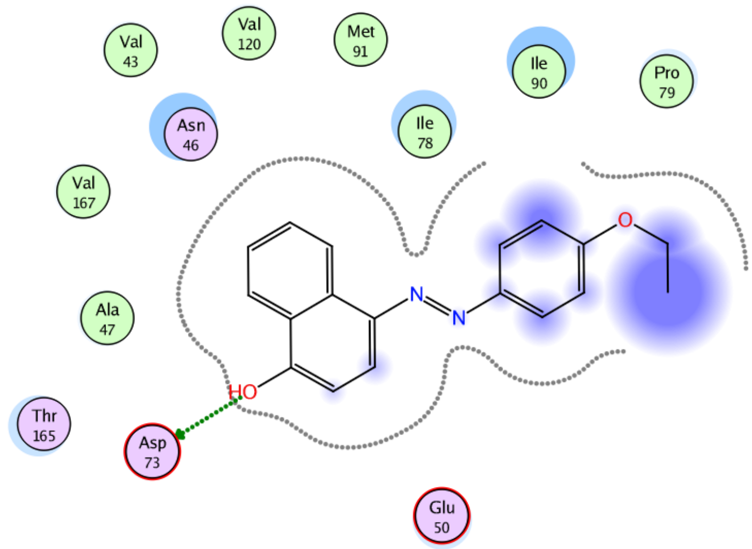
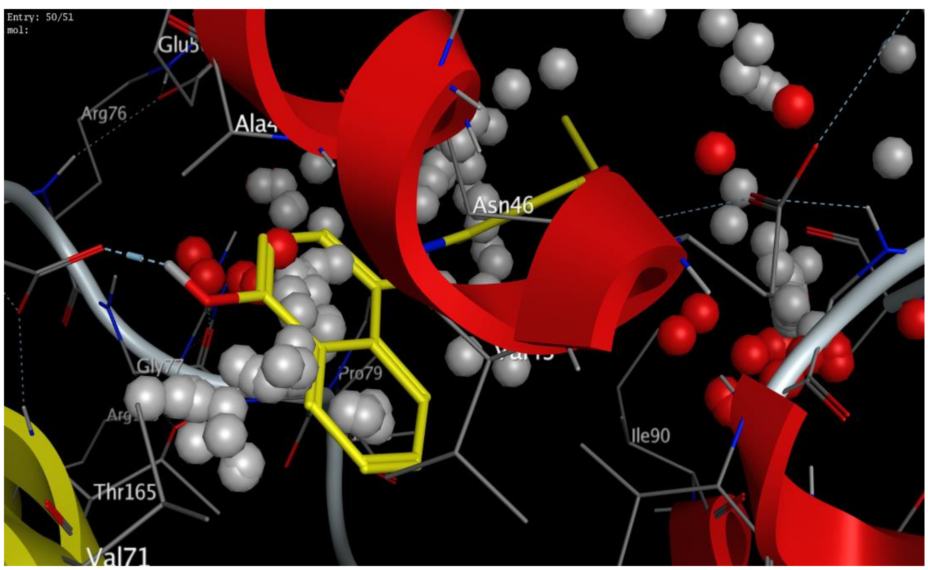
Pro79
Ile90
Met91

4.2h





Fat brown B

Figure 4.9: 2D and 3D binding interaction of target compounds, **4.2h**, **4.2b** and fat brown B inside the enzyme**.**

The results of the docking experiments were summarized in **Table 4.5**. The 2D and 3D binding map of the target compounds **4.2b** and **4.2h** in the pocket is explained through three different fragments around the chromene scaffold, 8-NH_2, 9-CN and -Br belong to **4.2h**. These fragments form stable hydrogen bonding interactions with a panel of corresponding pocket residues: Asp 73, Thr 165, Asn 46, Asp 49 and Lie 78 with different distance between 2.00 and 3.23 Å.

Compound No.		**4.2b**	**4.2h**	**Fat Brown B**
Amino acid (Distance Å)	**Asp 73**	-NH_2 (2.3, 2.4)	-	--OH (2.4)
	Thr 165	-CN (3.2)	-	-
	Asn 46	-	--NH_2 (2.0)	-
	Asp 49	-	--Br (3.2)	-
	Lie 78	-	-phenyl	
Interaction type		H-bonding	H-bonding (aromatic)	H-bonding
ΔG (kcal/mol)		-13.61	-12.23	-11.24

Table 4.5: Description of the docking data of the selected target compounds

The data reported in the table are extracted from MOE program showing the corresponding amino acids residues in enzyme pocket, corresponding fragments of ligands, interaction distances, types of interaction, and their binding energy to some selected drugs compared to reference drug.

4.4 Conclusions

We have synthesized a series of 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4*H*-benzo [*h*] chromene-3-carbonitrile derivatives **4.2(a-j)** based on 1-naphthalenol-4- [(4-ethoxy phenyl) azo] **4.1** by reaction of benzaldehyde derivatives and malononitrile or ethyl cyanoacetate in an ethanol and few drops of piperidine (Knoevenagel condensation) followed by the electrophilic substitution step of 1-naphthalenol-4-[(4-ethoxyphenyl) azo] **4.1**(Michael addition adduct). All synthesized compounds were characterized by IR and NMR. The antimicrobial activity of synthesized derivatives showed greater than the reference drugs against fungal species and Gram-negative bacteria and slight activity towards Gram-positive bacteria. The novel compounds were found to have comparable or slightly greater cytotoxicity effects compared to the reference compounds. Compounds **4.2e**, **4.2h** and **4.2j** have good IC_{50} ranging from 4.35 to 5.54 µg/mL against HCT-116 and MCF-7 cell lines. Moreover, all the target derivatives showed less activity in case of HepG-2 cell line. The docking study results showed binding interaction of synthesized compounds **3.2b**, **3.2h** in the active site of enzyme.

4.5 Experimental Section

4.5.1 Materials and Instrumentation

Chemicals and solvents were purchased from Sigma-Aldrich (Canada) and Alfa Aesar, and were used as received. Compound **4.1** was purchased from Sigma-Aldrich (Canada). Melting points were determined in open capillaries using an electrothermal apparatus and are uncorrected. The progress of the reactions was monitored using thin layer chromatography (TLC) on Merck silica gel 60 F254 plates. Infrared (IR) spectra were recorded using Bruker Alpha FT-IR Spectrometer as pressed KBr pellets. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 300 MHz and at 75 MHz, respectively, on a Bruker Avance Spectrometer in DMSO-d_6 with tetramethylsilane as internal standard. The high vacuum for overnight and heat gun were used to removed residual water from the compounds. Elemental analyses for C, H and N were performed using an Exeter Analytical, Inc. CE-440 Elemental Analyzer. UV-vis absorption measurements were performed using a HP8543 UV-vis spectrophotometer.

4.5.2 Biological Studies

4.5.2.1 *Antimicrobial Screening*

The microorganism inoculums were uniformly spread using sterile cotton swabs on a sterile Petri dish malt extract agar (for fungi) and nutrient agar (for bacteria). One hundred cubic millimeters of each sample was added to each well (10-mm-diameter holes were cut in the agar gel, 20 mm apart from one another). The systems were incubated for 24–48 hr. at 37ºC (for bacteria) and at 28ºC (for fungi). After incubation, microorganism growth was observed. Inhibition zones of the bacterial and fungal growth were measured in millimeters. Tests were performed in triplicate. [113,114]

4.5.2.2 *Cytotoxic Screening*

Human colon carcinoma (HCT-116), human hepatocellular carcinoma (HepG-2), adenocarcinomic and human breast adenocarcinoma (MCF-7) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/mL gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. Potential cytotoxicity of the compounds was evaluated on tumor cells using the method of Gangadevi and Muthumary.[130] The cells were grown as monolayers in growth RPMI-1640. The monolayers of 104 cells adhered at the bottom of the wells in a 96-well microliter plate incubated for 24 h at 37ºC in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M pH 7.2) and simultaneously the cells were treated with 100 µL from different dilutions of tested sample in fresh maintenance medium and incubated at 37 ºC. A control of untreated cells was made in the absence of tested sample. Positive controls containing doxorubicin were also tested as a reference drug for comparison.

Six wells were used for each concentration of the test sample. Every 24-hour observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing using 33% glacial acetic acid and the absorbance read at 590 nm using a microplate reader (SunRise, TECAN, Inc, USA) through mixing. [121,131] The absorbance values from untreated cells were considered as 100% proliferation. The number of viable cells was determined using microplate reader as previously mentioned and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration was plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50 % inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

4.5.3 Molecular modeling

The newly synthesized compounds were docked into the crystal structure of *E. coli* topoisomerase II DNA gyrase B (PDB code 1KZN). The MOE software[124] was used for all docking calculations. The MOE tools package was employed to generate the docking input files and to analyze the docking results. All non-polar hydrogens, clorobiocin, and crystal water molecules were removed prior to the calculations and the protonation of the enzyme was carried out and was energy minimized.

In each case, 100 docked structures were generated using genetic algorithm searches. The 3D structures of new synthesized compounds were drawn in MOE and the protonation of ligands were carried out. The energy of compounds was minimized up to 0.05 gradient using GBVI/WSA dG force field. These data were saved in database as input file MOE. Heavy atom comparison root means square deviations (RMSD values) were calculated and initial ligand binding modes were plotted. Protein-ligand interaction plots were generated using MOE 2012.10.

4.5.4 Synthesis

4.5.4.1 General procedure for the synthesis of 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4H-benzo[h]chromenederivatives

A mixture of compound **4.1** (2.3 mmol), malononitrile or ethyl cyanoacetate (2.3 mmol), and aryl aldehyde (2.3 mmol) in ethanol (5 mL) and few drops of pipredine was added. The reaction mixture was stirred under reflux. After completion of the reaction (monitored by TLC), the mixture was cooled and filtered, washed with ethanol and hexane to afford the **4.2(a-j)**.

4.5.4.2 2-amino-6-(4-ethoxyphenylazo)-4-(2-fluoro-phenyl)-4H-benzo [h] chromene-3-carbonitrile (4.2a)

Brown solid (0.48g, 62.05 %), m.p. 205°C; IR (KBr) cm^{-1}: 3469, 3280 (NH_2), 2925, 2891 (CH), 2201 (CN), 1571 (N=N); ^1H NMR (300 MHz, DMSO) δ 8.87 – 8.83 (m, 1H, Ar-H), 8.39 – 8.34 (m, 1H, Ar-H), 7.94 (d, J = 8.9 Hz, 2H, Ar-H), 7.81 – 7.77 (m, 2H, Ar-H), 7.41

(s, 1H, Ar-H), 7.37 – 7.28 (m, 4H, Ar-H, NH_2), 7.22 – 7.14 (m, 2H, Ar-H), 7.10 (d, J = 9.0 Hz, 2H, Ar-H), 5.27 (s, 1H, H4), 4.14 (q, J = 6.7 Hz, 2H, CH_2), 1.36 (t, J = 6.8 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 162.30 (C-2), 160.96, 147.53, 146.07, 143.96, 132.72 (Ar-C), 132.55, 131.35 (Ar-CH), 131.15 (Ar-C), 130.35, 128.81 (Ar-CH), 128.32 (Ar-C), 125.76, 124.11, 121.98, 120.85 (Ar-CH), 117.80 (CN), 116.95 (Ar-C), 116.67, 115.90, 112.18 (Ar-CH), 64.57 (CH_2), 55.67 (C-3), 31.52 (CH-4), 15.39 (CH_3); Anal. Calcd for $C_{28}H_{21}N_4O_2F$: C, 72.40; H, 4.56; N, 12.06. Found: C, 72.05; H, 4.50; N, 11.87.

4.5.4.3 2-amino-6-(4-ethoxyphenylazo)-4-(2-chloro-phenyl)- 4H-benzo [h] chromene-3-carbonitrile (4.2b)

Greenish-brown solid (0.60g, 74.92%), m.p. 219°C; IR (KBr) cm^{-1}: 3466, 3326 (NH_2), 2995, 2973, 2932 (CH), 2197 (CN), 1567 (N=N); ^{1}H NMR (300 MHz, DMSO) δ 8.81-8.79 (m, 1H, Ar-H), 8.37-8.34 (m, 1H, Ar-H), 7.90 (d, J = 8.8 Hz, 2H, Ar-H), 7.78 – 7.75 (m, 2H, Ar-H), 7.45 (d, J = 7.1 Hz, 1H, H), 7.40-7.21 (m, 6H, Ar-H,NH_2), 7.07 (d, J = 8.8 Hz, 2H, Ar-H), 5.46 (s, 1H, H4), 4.11 (q, J = 6.7 Hz, 2H, CH_2), 1.35 (t, J = 6.8 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 162.32 (C-2), 160.86, 147.49, 146.03, 143.92, 142.72 (Ar-C), 133.01, 132.22, 131.38 (Ar-CH), 130.93 (Ar-C), 129.98, 128.87, 128.35 (Ar-CH), 125.78 (Ar-C), 124.07, 123.97, 122.03, 120.71 (Ar-CH), 117.69 (CN), 115.91 (Ar-CH), 111.87 (Ar-C), 64.57 (CH_2), 55.78 (C-3), 31.53 (CH-4) 15.39 (CH_3); Anal. Calcd for $C_{28}H_{21}N_4O_2Cl$: C, 69.92; H, 4.40; N, 11.65. Found: C, 70.25; H, 4.20; N, 11.76.

4.5.4.4 2-amino-6-(4-ethoxyphenylazo)-4-(2-bromo-phenyl)- 4H-benzo [h] chromene-3-carbonitrile (4.2c)

Brown solid (0.51g, 58.29 %), m.p. 208°C; IR (KBr) cm^{-1}: 3468, 3326 (NH_2), 2995, 2973, 2932 (CH), 2198 (CN), 1569 (N=N); ^{1}H NMR (300 MHz, DMSO) δ 8.86 – 8.80 (m, 1H, Ar-H), 8.39 – 8.34 (m, 1H, Ar-H),, 7.92 (d, *J* = 9.0 Hz, 2H, Ar-H), 7.80 – 7.76 (m, 2H, Ar-H), 7.64 (d, *J* = 7.5 Hz, 1H, Ar-H), 7.40 – 7.27 (m, 5H, Ar-H, NH_2), 7.25 – 7.19 (m, 1H, Ar-H), 7.09 (d, *J* = 9.0 Hz, 2H, Ar-H), 5.49 (s, 1H, H4), 4.13 (q, *J* = 6.9 Hz, 2H, CH_2), 1.36 (t, *J* = 6.9 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 162.32 (C-2), 160.78, 147.46, 145.89, 143.91 (Ar-C), 134.10, 132.45, 131.39, 130.24 (Ar-CH), 129.54 (Ar-C), 128.86, 128.34, 125.78 (Ar-CH), 124.11(Ar-C), 123.96 (Ar-CH), 123.32 (Ar-C), 122.06, 120.65 (Ar-CH), 117.80 (CN), 115.89 (Ar-CH), 111.75 (Ar-C), 64.56 (CH_2), 56.01 (C-3), 31.53 (CH-4), 15.38 (CH_3); Anal. Calcd for $C_{28}H_{21}N_4O_2Br$: C, 64.01; H, 4.03; N, 10.66. Found: C, 64.32; H, 3.78; N, 10.78.

4.5.4.5 2-amino-6-(4-ethoxyphenylazo)-4-(3-bromo-phenyl)- 4H-benzo [h] chromene-3-carbonitrile (4.2d)

Brown solid (0.53g, 60.57 %), m.p. 217°C; IR (KBr) cm^{-1}: 3466, 3335 (NH_2), 2975, 2935, 2883 (CH), 2193 (CN), 1566 (N=N); ^{1}H NMR (300 MHz, DMSO) δ 8.89 – 8.80 (m, 1H, Ar-H), 8.37 – 8.36 (m, 1H, Ar-H),, 7.95 (d, *J* = 9.0 Hz, 2H, Ar-H), 7.81-7.77 (m, 2H, Ar-H), 7.50 (s, 1H, Ar-H), 7.46-7.42 (m, 2H, Ar-H), 7.34 – 7.29 (m, 4H, Ar-H, NH_2), 7.12 (d, *J* = 9.0 Hz, 2H, Ar-H), 5.09 (s, 1H, H4), 4.15 (q, *J* = 6.9 Hz, 2H, CH_2), 1.37 (t, *J* = 6.9 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ162.33 (C-2), 160.68, 149.15, 147.55, 145.82 (Ar-C), 144.06, 132.04, 131.33 (Ar-CH), 131.20 (Ar-C), 130.94, 128.88, 128.36 (Ar-CH), 127.83 (Ar-C), 125.78, 124.22 (Ar-CH), 122.89 (Ar-C), 122.05, 120.89 (Ar-CH), 118.38 (CN), 115.93 (Ar-CH), 112.48 (Ar-C), 64.58 (CH_2), 56.89 (C-3), 31.53 (CH-4), 15.40 (CH_3) Anal. Calcd for $C_{28}H_{21}N_4O_2Br$: C, 64.01; H, 4.03; N, 10.66. Found: C, 64.27; H, 3.77; N, 10.80.

4.5.4.6 2-amino-6-(4-ethoxyphenylazo)-4-(3-chloro-phenyl)- 4H-benzo [h] chromene-3-carbonitrile (4.2e)

Dark brown solid (0.54 g, 67.43 %), m.p. 212°C; IR (KBr) cm^{-1}: 3470, 3336 (NH_2), 2979, 2927, 2875 (CH), 2194 (CN), 1566 (N=N); ^{1}H NMR (300 MHz, Acetone) δ 8.97-8.90 (m, 1H-Ar-H), 8.44-8.38 (m, 1H-Ar-H), 7.98 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.80 – 7.73 (m, 2H, Ar-H), 7.55 (s, 1H, Ar-H), 7.43-7.27 (m, 4H, Ar-H, NH_2), 7.15 (d, *J* = 11.7 Hz, 1H, Ar-H), 7.12 (d, *J* = 9.0 Hz, 2H, Ar-H), 6.62 (s, 1H, Ar-H), 5.11 (s, 1H, H4), 4.19 (q, *J* = 7.0 Hz, 2H, CH_2), 1.43 (t, *J* = 7.0 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, Acetone) δ 162.40 (C-2), 148.39, 147.68, 144.49, 134.54, 131.60 (Ar-C), 130.95, 128.32 (Ar-CH), 128.10 (Ar-C), 127.70, 127.05, 125.34 (Ar-CH), 125.16 (Ar-C), 124.34, 123.77, 122.49, 121.60, 119.23 (Ar-CH), 117.95 (CN), 115.28 (C-Ar-CH), 112.22 (Ar-C), 64.14 (CH_2), 58.49 (C-3), 41.71 (CH-4), 14.48 (CH_3); Anal. Calcd for $C_{28}H_{21}N_4O_2Cl$: C, 69.92; H, 4.40; N, 11.65. Found: C, 70.17; H, 4.21; N, 11.70.

4.5.4.7 2-amino-6-(4-ethoxyphenylazo)-4-(4-fluoro-phenyl)- 4H-benzo [h] chromene-3-carbonitrile (4.2f)

Greenish-brown solid (0.43g, 55.58 %), m.p. 214°C; IR (KBr) cm^{-1}: 3466, 3278 (NH_2), 2979, 2935, 2869 (CH), 2187 (CN), 1567 (N=N); ^{1}H NMR (300 MHz, DMSO) δ 8.86 – 8.81 (m, 1H, Ar-H), 8.38 – 8.35 (m, 1H, Ar-H), 7.95 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.81 – 7.76 (m, 2H, Ar-H), 7.41 (s, 1H, Ar-H), 7.35 – 7.27 (m, 4H, Ar-H, NH_2), 7.19 – 7.09 (m, 4H, Ar-H), 5.07 (s, 1H, H4), 4.15 (d, *J* = 7.0 Hz, 2H, CH_2), 1.37 (t, *J* = 6.9 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 162.31(C-2), 160.51, 147.54, 145.74, 143.99, 142.69, 131.26 (Ar-C), 130.59, 130.48 (Ar-CH), 128.81 (Ar-C), 128.32, 125.76, 125.24 (Ar-CH), 124.21 (Ar-C), 123.98, 122.02 (Ar-CH), 120.99 (Ar-C), 118.92 (CN), 116.61, 116.32, 115.92, 112.60 (Ar-CH), 64.57 (CH_2), 57.34 (C-3), 31.54 (CH-4), 15.40 (CH_3); Anal. Calcd for $C_{28}H_{21}N_4O_2F$: C, 72.40; H, 4.56; N, 12.06. Found: C, 72.16; H, 4.32; N, 11.80

4.5.4.8 2-amino-6-(4-ethoxyphenylazo)-4-(3-nitro-phenyl)- 4H-benzo [h] chromene-3-carbonitrile (4.2g)

Brown solid (0.54g, 65.99 %), m.p. 216°C; IR (KBr) cm^{-1}: 3418, 3331 (NH_2), 2980, 2967, 2923 (CH), 2210 (CN), 1571 (N=N), 1473, 1352 (NO_2); ^{1}H NMR (300 MHz, DMSO) δ 8.85 – 8.81 (m, 1H, Ar-H), 8.41 – 8.37 (m, 1H, Ar-H), 8.18 (s, 1H, Ar-H), 8.12 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.92 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.82 – 7.76 (m, 3H, Ar-H), 7.64 (d, *J* = 7.8 Hz, 1H, Ar-H), 7.45-7.41 (m, 3H, Ar-H, NH_2), 7.09 (d, *J* = 8.9 Hz, 2H, Ar-H), 5.32 (s, 1H, H4), 4.12 (q, *J* = 6.8 Hz, 2H, CH_2), 1.35 (t, *J* = 6.9 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 162.33 (C-2), 160.83, 148.91, 148.58, 147.50, 145.91, 144.16 (Ar-C), 135.47, 131.47 (Ar-CH), 131.39 (Ar-C), 128.96, 128.40, 125.75 (Ar-CH), 124.23 (Ar-C), 124.02, 123.14, 122.98 (Ar-CH), 122.07 (Ar-C), 120.80 (Ar-CH), 117.99 (CN), 115.89, 112.43 (Ar-CH), 64.56 (CH_2), 56.56 (C-3), 31.52 (CH-4), 15.38 (CH_3); Anal. Calcd for $C_{28}H_{21}N_5O_4$: C, 68.42; H, 4.31; N, 14.25. Found: C, 68.73; H, 4.20; N, 14.02.

4.5.4.9 Ethyl-2-amino-6-(4-ethoxyphenylazo)-4-(2-bromo-phenyl)-4H-benzo[h]chromene -3-carboxylate (4.2h)

Dark brown solid (0.56g, 58.43 %), m.p. 145.2°C; IR (KBr) cm^{-1}: 3451, 3296 (NH_2), 2981, 2939, 2885 (CH), 1674 (CO), 1516 (N=N); ^{1}H NMR (300 MHz, DMSO) δ 8.80 (dd, *J* = 5.9, 2.5 Hz, 1H, Ar-H), 8.42 (dd, *J* = 6.2, 3.4 Hz, 1H, Ar-H), 7.91 (m, 4H, Ar-H, NH_2), 7.79 – 7.71 (m, 2H, Ar-H), 7.67 (s, 1H, Ar-H), 7.56 (d, *J* = 7.7 Hz, 1H, Ar-H), 7.27 – 7.19 (m, 2H, Ar-H), 7.11-7.05 (m, 3H, Ar-H), 5.64 (s, 1H, H4), 4.12 (q, *J* = 6.8 Hz, 2H, CH_2), 3.51 (s, 3H, OCH_3), 1.36 (t, *J* = 6.6 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 169.22 (C=O), 168.92, 162.25 (Ar-C), 161.22 (C-2), 147.61, 147.47, 145.45 (Ar-C), 143.94, 133.50, 131.21, 129.24 (Ar-CH), 129.12 (Ar-C), 128.58, 128.15, 125.72 (Ar-CH), 124.20 (Ar-C), 123.16 (Ar-CH), 122.96 (Ar-C), 122.07 (Ar-CH), 120.94 (Ar-C), 115.89, 111.92 (Ar-CH), 76.29 (C-3), 64.55 (CH_2), 51.36 (OCH_3), 28.16 (CH-4), 15.38 (CH_3); Anal. Calcd for $C_{29}H_{24}N_3O_4Br$: C, 62.94; H, 4.58; N, 7.34. Found: C, 63.25; H, 4.32; N, 7.62.

4.5.4.10 Ethyl-2-amino-6-(4-ethoxyphenylazo)-4-(2-chloro-phenyl)-4H-benzo[h]chromene -3-carboxylate (4.2i)

Light brown solid (0.45g, 50.78 %), m.p. 139.9-140°C; IR (KBr) cm^{-1}: 3454, 3316 (NH_2), 2977, 2934 (CH), 1675 (CO), 1535 (N=N); ^1H NMR (300 MHz, DMSO) δ 8.79-8.76 (m, 1H, Ar-H), 8.41 – 8.37 (m, 1H, Ar-H), 7.93 – 7.86 (m, 4H, Ar-H, NH_2), 7.75-7.67 (m, 2H, Ar-H), 7.56 (s, 1H, Ar-H), 7.37 (dd, J = 6.8, 1.10 Hz, 1H, Ar-H), 7.26 (ddd, J = 7.7, 3.7, 1.10 Hz, 1H, Ar-H), 7.20 (ddd, J = 6.6, 3.7, 1.7 Hz, 1H, Ar-H), 7.13 (dd, J = 7.6, 1.7 Hz, 1H, Ar-H), 7.05 (d, J = 9.0 Hz, 2H, Ar-H), 5.60 (s, 1H, H4), 4.08 (q, J = 6.9 Hz, 2H, CH_2), 3.52 (s, 3H, OCH_3), 1.33 (t, J = 6.9 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 207.36 (C=O), 169.18, 168.88, 162.24 (Ar-C), 161.36 (C-2), 147.49, 145.54, 143.93 (Ar-C), 132.27, 131.24, 131.19 (Ar-CH), 130.33 (Ar-C), 128.88, 128.58, 128.16, 125.81 (Ar-CH), 124.14 (Ar-C), 123.89, 122.05 (Ar-CH), 120.71 (Ar-C), 115.89, 112.05 (Ar-CH), 75.97 (C-3), 64.55 (CH_2), 51.38 (OCH_3), 32.97 (CH-4), 15.38 (CH_3); Anal. Calcd for $C_{29}H_{24}N_3O_4Cl$: C, 68.24; H, 4.96; N, 7.96. Found: C, 68.57; H, 4.73; N, 8.19.

4.5.4.11 Ethyl-2-amino-6-(4-ethoxyphenylazo)-4-(3-bromo-phenyl)-4H-benzo[h]chromene-3-carboxylate (4.2j)

Orange solid (0.75g, 78.26 %), m.p. 158.9°C; IR (KBr) cm^{-1}: 3489, 3346 (NH_2), 2976, 2949, 2871 (CH), 1675 (CO), 1487 (N=N); ^1H NMR (300 MHz, DMSO) δ 8.82 (dd, J = 5.5, 3.1 Hz, 1H, Ar-H), 8.43 (dd, J = 6.4, 3.5 Hz, 1H, Ar-H), 7.95 (d, J = 8.9 Hz, 2H, Ar-H), 7.89 (s, 2H, NH_2), 7.80 – 7.71 (m, 2H, Ar-H), 7.60 (s, 1H, Ar-H), 7.43 (s, 1H, Ar-H), 7.33-7.26 (m, 2H, Ar-H), 7.22-7.18 (m, 1H, Ar-H), 7.11 (d, *J* = 9.0 Hz, 2H, Ar-H), 5.16 (s, 1H, H4), 4.13 (q, *J* = 6.9 Hz, 2H, CH_2), 3.57 (s, 3H, OCH_3), 1.36 (t, *J* = 6.9 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 169.05 (C=O), 162.25 (Ar-C), 161.31 (C-2), 151.13, 147.57, 145.96, 144.14 (Ar-C), 131.64 (Ar-CH), 131.12 (Ar-C), 130.73, 130.08, 128.60, 128.18, 127.35, 125.72 (Ar-CH), 124.19 (Ar-C), 123.97 (Ar-CH), 122.51 (Ar-C), 122.05 (Ar-CH), 121.46 (Ar-C), 115.88, 112.89 (Ar-CH), 79.93 (C-3), 78.32 (CH_2), 57.67 (OCH_3), 39.95 (CH-4), 18.45 (CH_3); Anal. Calcd for $C_{29}H_{24}N_3O_4Br$: C, 62.94; H, 4.58; N, 7.34. Found: C, 63.20; H, 4.36; N, 7.54.

Chapter Five: Conclusions

Chromenes are one of many families of biologically active compounds which have been used in the treatment of different diseases for many years. A large number of chromene derivatives have many varying activities. In this thesis I designed and synthesized several new chromene derivatives based on three different moieties of biologically active compounds and *in vitro* evaluated their antimicrobial and cytotoxic activities to the development of new antibiotic and anticancer drugs.

The biological importance of coumarin scaffold and the study of some reported 4-hyroxy coumarin based natural products have encouraged me to design novel antimicrobial agents with high potency and target selectivity. A synthetic route to these novel 8-amino-10-phenyl-5-hydroxy-2-oxo-4-propyl-2*H*, 10*H*-pyrano [2,3-*f*] chromene-9-carbonitrile derivatives **2.2(a-j**) has been successfully achieved. The key step represents a condensation of benzaldehyde derivatives and malononitrile in an ethanol with few drops of piperidine (Knoevenagal condensation) followed by the electrophilic substitution step of 5,7-dihydroxy-4-propyl-chromen-2-one (Michael addition adduct) and producing the desired products. Fluorescence of the new compounds showed emission peaks at around 450 nm with good quantum yields. The new synthesized compounds were screened for testing their antimicrobial activity against seven bacteria, four fungi, and one mycobacterium. The activity data show that these novel compounds have potent antibacterial and antifungal activities compared to reference drugs.

The inhibition zones cover a good range of activity, with corresponding minimum inhibitory concentrations. Analysis of the mode of action through docking experiments was reported. In addition, a cytotoxic screening evaluation step was employed against four human cancer cell lines and the resulting inhibition was comparable or greater than the reference drug.

In this study I prepared novel 8-amino-10-phenyl-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-diydro-2*H*,10*H*-pyrano [2,3-*f*] chromene derivatives **3.2(a-g)** and evaluation of their antimicrobial activity and cytotoxic effects. These target compounds were synthesized by condensation of benzaldehyde derivatives and malononitrile in an ethanolic piperidine (Knoevenagal condensation) followed by the electrophilic substitution step of 4`5,7-trihydroxy-flavanone (Michael addition adduct). The structures of synthesized compounds were established from spectral data. The results of antibacterial assay revealed that the compounds **3.2a, 3.2b** and **3.2(d-g)** exhibited the highest inhibitory activity against P.A and *E. coli* by IZ range 18.3 to 24.6 mm and MIC 0.49 to 3.9 µg/mL compared to reference drugs. Compounds **3.2a**, **3.2b** and **3.2e** were found to be more effective against S.T by IZ 23.4-26.4 mm. The antibacterial activity of the most compounds was found to be comparably active to reference drugs against gram-positive bacteria. Moreover, the cytotoxic activity was also evaluated against four different human carcinoma cell lines. Most of these compounds have good inhibitory activity against HCT-116 cell line (IC_{50}= 1.08-1.48 µg/ml). Finally, compound **3.2c** showed no antimicrobial activity against any of the tested bacteria, fungi and TB.

The molecular modeling results showed binding interaction of synthesized compounds **3.2a**, **3.2b**, **3.2d**, **3.2e** and kaempferol in the active site of GyrB.

Additionally, synthesis of new several of 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4*H*-benzo [*h*] chromene-3-carbonitrile derivatives **4.2(a-j**) was reported in this thesis, which was based on 1-naphthalenol-4- [(4-ethoxy phenyl) azo] **4.1** by reacting of benzaldehyde derivatives and malononitrile or ethyl cyanoacetate in an ethanol and few drops of piperidine (Knoevenagal condensation) followed by the electrophilic substitution step of 1-naphthalenol-4-[(4-ethoxyphenyl) azo] **4.1**(Michael addition adduct). All synthesized compounds were characterized by IR and NMR. The antimicrobial activity of synthesized derivatives showed greater than the reference drugs against fungal species and Gram-negative bacteria and slightly activity towards Gram-positive bacteria. The novel compounds were found to be comparable or slightly cytotoxicity effects. Compounds **4.2e**, **4.2h** and **4.2j** have good IC_{50} ranging from 4.35 to 5.54 µg/mL against of HCT-116 and MCF-7 cell lines. Moreover, all the target derivatives showed less activity in case of HepG-2 cell line. The docking study results for new synthesized compounds **3.2b** and **3.2 h** was reported. Bothe of these two compounds have different interaction with different amino acid inside the gyrase B.

Finally, a protocol was developed combining chemistry and biology for design and synthesis of potent antimicrobial and cytotoxic effects of 8-amino-10-phenyl-5-hydroxy-2-oxo-4-propyl-2*H*, 10*H*-pyrano[2,3-*f*] chromene-9-carbonitrile derivatives **2.2 (a-j)**, 8-amino-10-phenyl-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-diydro-2*H*,10*H*-pyrano[2,3-

f] chrome-ne derivatives **3.2(a-g)** and 2-amino-6-(4-ethoxyphenylazo)-4(-phenyl)-4*H*-benzo [*h*] chromene-3-carbonitrile derivatives **4.2(a-j).** A series of novel chromene derivatives based on three different moieties of biologically active compounds were synthesized and their antibacterial and anti-cancer activities were evaluated *in vitro*. The molecular modeling results showed binding interaction of some new synthesized compounds in the active site of Gyrase B. Future study could focus on chromenes combination with coumarin and flavanone moieties due to they have more biological activity than azo dyes.

References

1. Gecn, G. R.; Evans, J. M.; Vong, A. K. *comprehensive, Heterocyclic Chemistry*, 1nd ed. Katritzky, A. R., Rccs, C. W.; Scrivcn, E. F. V.; Eds.; Pcrgamon: New York, **1996**, 469 - 500.
2. Van Otterlo, W.; Ngidi, E.; Kuzvidza, S.; Morgans, G.; Moleele, S.; Koning, C. Tetrahedron **2005**, *61*, 9996–10006.
3. Eichert, T.; Hauptmann, S.; Speichcr, A. *The Chemistry of Heterocycles: Structure, Reactions, Synthesis and Applications,* 2nd ed.John Wiley, **2004**
4. Murray, R.D. FortschrChem Org Naturst, **2002**, *83*, 1-619.
5. Khafagy, M.M.; El-Wahas, A.H.F.; Eid, F.A.; El-Agrody, A.M. Farmaco, **2002**, *57*, 715-722.
6. Smith, P.W.; Sollis, S.L.; Howes, P.D.; Cherry, P.C.; Starkey, I.D.; Cobley, K.N.; Weston, H.; Scicinski, J.; Merritt, A.; Whittington, A.; Wyatt, P.; Taylor, N.; Green, D.; Bethell, R.; Madar, S.; Fenton, R.J.; Morley, P.J.; Pateman, T.; Beresford, A.*J. Med. Chem*. **1998**, *41*, 787-797.
7. Martinez, A.G.; Marck, L.*J. Bioorg. Med. Chem. Lett*. **1997**, *7*, 3165-3170.
8. Nitin, K.; Sushil, K.; Himanshu, G.; Sharma, P.K. *WRJB*. **2012**, *1*, 1-5.
9. Milan, M.; Mirjana, M.; Desanka, B.; Sanja, M.; Neda, N.; Vladimir, M. *Int J Mol. Sci*. **2011**, *12*, 2822-41.
10. Dell, C.P.; Smith, C.W. *Chem. Abstr*. **1993**, *119*, 139102d.
11. Mohr, S.J.; Chirigios, M.A.; Fuhrman, F.S.; Pryor, J.W. *Cancer Res*. **1975**, *35*, 3750-3754.
12. Foye, W.O. *Principi di ChemicoFarmaceutica*, Piccin: Padora, Italy, 1991, 416.
13. Andreani, L.L.; Lapi, E. *Bull. Chim.Farm.***1960**, *99*, 583-586.
14. Zhang, Y.L.; Chen, B.Z.; Zheng, K.Q.; Xu, M.L.; Lei, X.H.; Yaoxue, X.B. *ChemAbstr*. **1982**, *96*, 135383e.
15. Jain, N.; Xu, J.; Kanojia, R.M.; Du, F.; Jian-Zhong, G.; Pacia, E.; Lai, M.; Musto, A.; Allan, G.; Reuman, M.; Li, X.; Hahn, D.; Cousineau, M.; Peng, S.; Ritchie, D.; Russell, R.; Lundeen, S.; Sui, Z. *J. Med. Chem*. **2009**, *52*, 7544–7569.

16. Suresh, T.; Arunima, V.; Atin, K.; Sandeep, G.; Prarthana, V.R.; Ganesh, R.K. *Acta Pol. Pharm*. **2010**, *67*, 423-427.
17. Denish, C.K.; Hetal, K.P.; Nilesh, K.G. *AJBPR*. **2012**, *2*, 126-130.
18. Eiden, F.; Denk, F. *Arch. Pharm*. **1991**, *324*, 353-354.
19. Soh, N.; Sakawaki, O.; Makihara, K.; Odo, Y.; Fukaminato, T.; Kawai, T.; Irie, M.; Imato, T. *Bioorg. Med. Chem*. **2005**. *13*, 1131-1139.
20. Moorthy, J.N.; Venkatakrishnan, P.; Sengupta, S.; Baidya, M. *Organic letters,***2006**, *8*, 4891-4894.
21. Amr, A.G. E.; Mohamed, A. M.; Mohamed, S. F.; Abdel-Hafez, N.A.; Hammam, A.E. *Bioorg. Med. Chem.***2006**, *14*, 5481-5488.
22. Paliwal, P.K.; Jetti, S.R.; Jain, S. *Med. Chem. Res*. **2013**, *22*, 2984-2990.
23. Bhavanarushi, S.; Kanakaiah, V.; Yakaiah, E.; Saddanapu, V.; Addlagatta, A.; Rani, V.J. *Med. Chem. Res*. **2013**, *22*, 2446-2454.
24. Erichsen, M.N.; Huynh, T.H.; Abrahamsen, B.; Bastlund, J.F.; Bundgaard, C.; Monrad, O.; Jensen, A.B.; Nielsen, C.W.; Frydenvang, K.; Jensen, A.A.; Bunch, L.*J. Med. Chem*. **2010**, *53*, 7180-7191.
25. Kemnitzer, W.; Drewe, J.; Jiang, S.; Zhang, H.; Grundy, C.C.; Labreque, D.; Bubenick, M.; Attardo, G.; Denis, R.; Lamothe, S.; Gourdeau, H.; Tseng, B.; Kasibhatla, S.; Cai, S. *J. Med. Chem*. **2008**, *51*, 417-423.
26. Gao, Y.; Yang, W.; Du, D. *Tetrahedron: Asymmet,***2012**, *23*, 339–344.
27. Wiener, C.; Schroeder, C.H.; West, B.D.; Link, K.P. *J. Org. Chem*. **1962**, *27*, 3086-3088.
28. Ballini, R.; Bosica, G.; Conforti, M.L.; Maggi, R.; Mazzacani, A.; Righi, P.; Sartori, G. *Tetrahedron,***2001**, *57*, 1395-1398.
29. Shestopalov, A.M.; Emelianova, Y.M.; Nesterov, V.N.*Russian Chemical Bulletin, International Edition,***2002**, *51*, 2238-2243.
30. Jin, T.S.; Xiao, J.C.; Wang, S.J.; Li, T.S. *Ultrason. Sonochem.***2004**, *11*, 393–397.
31. Kidwai, M.; Saxena, S.; Khan, M.K.; Thukral, S.S. *Bioorg. Med. Chem. Lett*. **2005**, *15*, 4295–4298.

32. Yadav, J.S.; Reddy, B.V.; Gupta, M.K.; Prathap, I.; Pandey, S.K. *Catalysis Communications*,**2007**, *8*, 2208-2211.

33. Heravi, M.M.; Baghernejad, B.; Oskooie, H.A. *Journal of the Chinese Chemical Society*, **2008**, *55*, 659-662.

34. Makarem, S.; Mohammadi, A.A.; Fakhari, A.R. *Tetrahedron Letters*, **2008**, *49*, 7194-7196.

35. Raghuvanshi, D.S.; Singh, K.N. *ARKIVOC*. **2010**, *10*, 305-317.

36. Murthy, S.N.; Madhav, B.; Reddy, V.P.; Madhav, B.; Nageswar, Y.V. *Tetrahedron Letters*, **2010**, *51*, 3649-3653.

37. Khurana, J.M.; Nand, B.; Saluja, P. *Tetrahedron*, **2010**, *66*, 5637-5641.

38. Kolla, S.R.; Lee, Y.R. *Tetrahedron*, **2011**, *67*, 8271-8275.

39. Sabry, N.M.; Mohamed, H.M.; Khattab, E.S.; Motlaq, S.S.; El-Agrody, A.M. *European Journal of Medicinal Chemistry*, **2011**, *46*, 765-772.

40. Babu, T.H.; Kamalraja, J.; Muralidharan, D.; Perumal, P.T. *Tetrahedron Letters*, **2011**, *52*, 4093-4096.

41. Mehrabi, H.; Kazemi-Mireki, M. *Chinese Chemical Letters*,**2011**, *22*, 1419-1422.

42. Sheibani, H.; Saidi, K.; Abbasnejad, M.; Derakhshani, A.; Mohammadzadeh, I. *Arabian Journal of Chemistry*, 2011, *Available online 17 September 2011, In Press*

43. Gupta, A.K.; Kumari, K.; Singh, N.; Raghuvanshi, D.S.; Singh, K.N. *Tetrahedron Lett*. **2012**, *53*, 650–653.

44. Safari, J.; Heydarian, M.; Zarnegar, Z. *Arabian Journal of Chemistry*, **2013**, *Available online 27 November 2013, In Press.*

45. Sinha, S.; Ikoner, R.R; Kumar, S.; Mathew, J.; Roy, A.; Mukhopadhyay, S.K.; Nandi, C.K.; Ghosh, S. *Journal of Luminescence*, **2013**, *143*, 355–360.

46. Essamlali, Y.; Amadine, O.; Maati, H.; Abdelouahdi, K.; Fihri, A.; Zahouily, M.; Varma, R. S.; Solhy, A. *ACS Sustainable Chem. Eng*. **2013**, *1*, 1154−1159.

47. Albadi, J.; Mansournezhad, A.; Darvishi-Paduk, M. *Chinese Chemical Letters*, **2013**, *24*, 208-210.

48. Kiyani, H.; Ghorbani, F. *Journal of Saudi Chemical Society*, **2014**, *18*, 689-701.

49. Rajesh, U.C.; Kholiya, R.; Thakur, A.; Rawat, D.S. *Tetrahedron Letters*, **2015**, *56*, 1790-1793.

50. Massoor, S.S.; Logaiya, K.; Aswin, K.; Sudhan, P.N. *Journal of Taibah University for Science*, **2015**, *9*, 213-226.

51. El-Agrody, A.M.; Halawa, A.H.; Fouda, A.M.; Al-Dies, A.M. *Journal of Saudi Chemical Society*, **2016,** *Available online 19 March 2016*, *In Press*

52. Elinson, M.N.; Vereshchagin, A.N.; Bobrovsky, S.I.; Nasybullin, R.F.; Ilovaisky, A.I.; Merkulova, V.M. *C.R. Chimie*, **2016**, *19*, 293-298.

53. Jain, P.K.; Joshi, H. *Journal of Applied Pharmaceutical Science*, **2012**, *2*, 236-240.

54. Venugopala, K.N.; Rashmi, V.; Odhav, B. *BioMed Research International*, **2013**, *1*, 1-14.

55. Constantinou, A.I.; Kamath, N.; Hurley, J.S. *Eur. J. Can*. **1998**, *14*, 19-27.

56. Finn, G.; Kenealy, E.; Creaven, B.; Egan, D. *Cancer Letters*, **2002**, *18*, 54-61.

57. Pinheiro, P.F.; Justino, G.C. *Phytochemicals*,**2012**, *1*, 33-56.

58. Hwu, J.R.; Singha, R.; Hong, S.C.; Chang, Y.H.; Das, A.R.; Vliegen, I.; De Clercq, E.; Neyts, J. *Antiviral Res*, **2008**, 77, 157-162.

59. Egan, D.; James, P.; Cooke, D.; O'Kennedy, R. *Cancer Lett*, **1997**, 118, 201-211.

60. Valenti, P.; Rampa, A.; Recanatini, M.; Bisi, A.; Belluti, F.; Da Re, P.; Carrara, M.; Cima, L. *Anticancer Drug Des*, **1997**, 12, 443-451

61. Sardari, S.; Mori, Y.; Horita, K.; Micetich, R.G.; Nishibe, S.; Daneshtalab, M. *Bioorganic & medicinal chemistry*, **1999**, 7, 1933-1940.

62. Fylaktakidou, K.C.; Hadjipavlou-Litina, D.J.; Litinas, K.E.; Nicolaides, D.N. *Curr Pharm Des*, **2004**, 10, 3813-3833.

63. Ghate, M.; Manohar, D.; Kulkarni, V.; Shobha, R.; Kattimani, S.Y. *European journal of medicinal chemistry*, **2003**, 38, 297-302.

64. Reutrakul, V.; Leewanich, P.; Tuchinda, P.; Pohmakotr, M.; Jaipetch, T.; Sophasan, S.; Santisuk, T. *Planta Med*, **2003**, 69, 1048-1051.

65. Fraga, C.G.; Galleano, M.; Verstraeten, S.V.; Oteiza, P.I. *Molecular Aspects of Medicine*, **2010**, *31*, 435-445.

66. Gomes, A.; Fernandes, E.; Lima, J.L.; Mira, L.; Corvo, M.L. *Current Medicinal Chemistry*, **2008**, *15*, 1586-1605.

67. Spencer, J.P.; Vauzour, D.; Rendeiro, C. *Archives of Biochemistry and Biophyics*, **2009**, *492*, 1-9.

68. Spencer, J.P. *British Journal of Nutrition*, **2010**, *31*, 546-557.

69. Mohamed, G.A.; Ibrahim, S.R.; Elkhyat, E.; Ross, S.A.; Sayed, H.M.; El-Moghazy, S.A.; El-Shanawany, M.A. *Phytochemistry Letter*, **2015**, *11*, 177-182.

70. Ajani, O.O.; Akinremi, O.E.; Ajani, A.O.; Edobor-Osoh, A.; Anake, W.U. *Physical Review & Research International*, **2013**, *3*, 28-41.

71. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. *Am J ClinNutr*.**2004**, *79*, 727-747.

72. Dabbagh, H.A.; Teimouri, A.; Chermahini, A.N. *Dyes and Pigments*, **2007**, *73*, 239-244.

73. Asniza, M.; Issam, A.M.; Abdul Khalil, H.P.*Sains Malaysiana*, **2011**, *40*, 765–770.

74. Yager, K.G.; Barrett, C.J. *Azobenzene Polymers For Photonic Applications*; Zhao,Y.; Ikeda, T. Eds.; Smart Light-Responsive Materials: John Wiley & Sons, Inc. Hoboken, NJ, USA, 2008, pp 1-46.

75. Cho, Il-H.; Zoh, K.D.*Dyes and Pigments*, **2007**, *75*, 533-543.

76. Bortolus, P.; Gleria, M. *Journal of Inorganic and Organometallic Polymers*, **1994**, *4*, 205-236.

77. Beev, K.S.; Beeva, K.N.; Sainov, S.H. *Three-Dimensional Television* Ozaktas, H.M.; Onural,L. Eds.; Signals and Communication Technology*:* New York, Springer Berlin Heidelberg**,** 2008, pp 557-598.

78. Rai, H.; Bhattacharya, M.; Singh, J.; Bansal, T.K.; Vats, P.; Banerjee, U.C. *Critical Review in Environmental Science and Technology*, **2005**, *35*, 219-238.

79. Saratale, R.G.; Saratale, G.D.; Chang, J.S.; Govindwar, S.P. *Journal of the Taiwan Institute of Chemical Engineers*, **2011**, *42*, 138-157.

80. Ventura-Camargo, B.C.; Marin-Morales, M.A. *Textiles and light industrial science and technology*, **2013**, *2*, 85-103.

81. Pandey, A.; Singh, P.; Iyengar, L. *International Biodeterioration& Biodegradation*, **2007**, *59*, 73-84.

82. Golka, K.; Kopps, S.; Myslak, Z.W. *Toxicology Letters*, **2004**, *151*, 203-210.

83. Robinson, T.; McMullan, G.; Marchant, R.; Nigam, P. *Bioresource Technology*, **2001**, *77*, 247-255.

84. Wainwright, M.*Dyes and Pigments*, **2008**, *76*, 582-589.

85. Weglarz, E; Gorecki, L *CHEMIK* **2012**, *66*, 1298-1307.

86. Levy, S.B. *The Antibiotic Paradox. How Miracle Drugs are Destroying the Miracle*. Plenum Publishing, New York., 1992.

87. Cassir, N.; Rolain, J. M.; Brouqui, P. *Frontiers in microbiology,* **2014**, *5*, 551.

88. Gouveia, F.L.; de Oliveira, R.M.; de Oliveira, T.B; da Silva, I.M.; do Nascimento, S.C.; de Sena, K.X.; de Albuquerque, J.F. *European journal of medicinal chemistry,* **2009**, *44*, 2038-2043.

89. Murray, R.D. *Fortschr Chem Org Naturst* **2002**, *83*, 1-619.

90. Lewis, K; Ausubel, F.M. *Nat Biotechnol* **2006**, *24*, 1504-1507.

91. Gellert, M.; O'Dea, M.H.; Itoh, T.; Tomizawa, J. *Proceedings of the National Academy of Sciences of the United States of America,* **1976**, *73*, 4474-4478.

92. Widelski, J.; Popova, M.; Graikou, K.; Glowniak, K.; Chinou, I. *Molecules* **2009**, *14*, 2729-2734.

93. Debeljak, Z.; Skrbo, A.; Jasprica, I.; Mornar, A.; Plecko, V.; Banjanac, M.; Medic-Saric, M. *Journal of chemical information and modeling,* **2007**, *47*, 918-926.

94. Kim, D.; Lee, I.; Jung, J.; Yang S.-I. *Archives of Pharmacal Research,* **1999**, *22*, 25-29.

95. Du, L.; Mahdi, F.; Jekabsons, M.B.; Nagle, D.G.; Zhou, Y.D. *J Nat Prod.* **2011**, *74*, 240-248.

96. Kulkarni, M.V.; Pujar, B.G.; Patil, V.D. *Studies on coumarins, II. Arch Pharm. (Weinheim)* **1983**, *316*, 15-21.

97. Cai, S.X.; Drewe, J.; Kasibhatla, S. *Current medicinal chemistry,* **2006**, *13*, 2627-2644.

98. Kemnitzer, W.; Drewe, J.; Jiang, S.; Zhang, H.; Crogan-Grundy, C.; Labreque, D.; Bubenick, M.; Attardo, G.; Denis, R.; Lamothe, S. , Gourdeau, H.; Tseng, B.; Kasibhatla, S.; Cai, S.X. *Journal of medicinal chemistry,* **2008**, *51*, 417-423.

99. Gourdeau, H.; Leblond, L.; Hamelin, B.; Desputeau, C.;. Dong, K; Kianicka, I.; Custeau, D.; Boudreau, C.; Geerts, L; Cai, S.X.; Drewe, J.; Labrecque, D.; Kasibhatla, S.; Tseng, B. *Molecular cancer therapeutics*, **2004**, *3*, 1375-1384.

100. Sangani, C.B.; Shah, N.M.; Patel, M.P.; Patel, R.G. *Journal of the Serbian chemical society*, **2012**, *77*, 1165-1174.

101. Mladenovic, M.; Mihailovic, M; Bogojevic, D.; Matic, S.; Niciforovic, N.; Mihailovic, V.; Vukovic, N.; Sukdolak, S.; Solujic, S. *International journal of molecular sciences*, **2011**, *12*, 2822-2841.

102. Cheng, J.F.; Ishikawa, A.; Ono, Y.; Arrhenius, T.; Nadzan, A. *Bioorg. Med. Chem. Lett*, **2003**, *13*, 3647-3650.

103. Thareja, S.; Verma, A.; Kalra, A.; Gosain, S.; Rewatkar, P.V.; Kokil, G.R. *Acta poloniae pharmaceutica,* **2010**, *67*, 423-427.

104. Jain, N.; Xu, J.; Kanojia, R.M.; Du, F.; Jian-Zhong, G.; Pacia, E.; Lai, M.T.; Musto, A.; Allan, G.; Reuman, M.; Li, X.; Hahn, D.; Cousineau, M.; Peng, S.; Ritchie, D.; Russell, R.; Lundeen, S.; Sui. Z. *Journal of medicinal chemistry,* **2009**, *52*, 7544-7569.

105. J. Mori, M. Iwashima, M. Takeuchi, H. Saito. Chemical & pharmaceutical bulletin, **2006**, *54*, 391-396.

106. Aliaa, M.K.; Manal, M.K.; Eman, K.A.; Heba, A.H.; *International Journal Pharmaceutical Research and Development*, **2012**, *4*, 310-322.

107. Denish, C.K.; Hetal, K.P.; Godvani, N.K. *AJBPR.* **2012**, *2*, 126-130.

108. Kamdar, N.R.; Haveliwala, D.D.; Mistry, P.T.; Patel, S.K. *Med Chem Res.* **2010**, *20*, 854-864.

109. Nitin, K.; Sushil, K.; Himanshu, G.; Sharma, PK. *WRJB* **2012**, 1, 1-5.

110. Bhat, M.A.; Siddiqui, N.; Khan, S.A. *Acta poloniae pharmaceutica*, **2008**, *65*, 235-239.

111. Soh, N.; Sakawaki, O.; Makihara, K.; Odo, Y.; Fukaminato, T.; Kawai, T.; Irie, M.; Imato, T. *Bioorganic & medicinal chemistry*, **2005**, *13*, 1131-1139.

112. Moorthy, J.N.; Venkatakrishnan, P.; Sengupta, S.; Baidya, M. *Organic letters*, **2006**, *8*, 4891-4894.

113. Voskressensky, L.G.; Festa, A.A.; Varlamov, A.V. *Tetrahedron*, **2014**, *70*, 551-572.

114. Hutanu, C.A.; Zaharia, M.; Pintilie, O. *Molecules*, **2013**, *18*, 2266-2280.

115. Rusu, E.; Airinei, A.; Tigoianu, R.I. *Rom. Biotechnol. Lett.* **2011**, *16*, 130-140.

116. http: //www.chemical-ecology.net/java/solvents.htm. Accessed 16/04/2016

117. D.A. Vanden-Berghe, A.J. Vlientinck. In: *Methods in Plant Biochemistry VI. Assays for Bioactivity*; Hostettmann, K., Ed.; Academic Press Inc: San Diego, United States, 1991.

118. Cappuccino, J.G.; Sherman, N. *Microbiology: A Laboratory Manual.* Benjamin-Cummings Pub Co, California, 1999.

119. Atta-ur-Rahman, M.I.C.; Thomsen, W.J. *Bioassay Techniques for Drug Development Bioassay Techniques for Drug Development.* Harwood Academic Publishers: Amsterdam, The Netherlands., 2001.

120. Smania, J.A.; Monache, F.D.; Smania, E.F.A.; Cuneo, R.S. *International Journal of Medicinal Mushrooms*, **1999**, *1*, 325-330.

121. Mosmann, T. *Journal of immunological methods*, **1983**, *65*, 55-63.

122. Alley, M.C.; Scudiero, D.A.; Monks, A.; Hursey, M.L.; Czerwinski, M.J.; Fine, D.L.; Abbott, B.J.; Mayo, J.G.; Shoemaker, R.H.; Boyd, M.R. *Cancer research*, **1988**, *48*, 589-601.

123. Kaushik, C.P.; Lal, K.; Kumar, A.; Kumar, S. *Med Chem Res*. **2014**, *23*, 2995-3004.

124. Molecular Operating Environment (MOE) Chemical Computing Group, Quebec, Canada. 2012; http://www.chemcomp.com. Accessed on 30/02/2013.

125. Abdel-Aziz, A.A; Asiri, Y.A.; Al-Agamy, M.H. *European journal of medicinal chemistry*, **2011**, *46*, 5487-5497.

126. Tavares, L.C.; Chiste, J.J.; Santos, M.G.; Penna, T.C. *Bollettino chimico farmaceutico*, **1999**, *138*, 432-436.

127. Masunari, A.; Tavares, L.C. *Bioorganic & medicinal chemistry*, **2007**, 15, 4229-4236.

128. Tavares, L.C.; Penna, T.C.; Amaral, A.T. *Bollettino chimico farmaceutico*, **1997**, *136*, 244-249.

129. Cronenberger, L.; Dolfin, B.; Pacheco, H. *Comptes rendus hebdomadaires des seances de l'Academie des sciences. Serie D: Sciences naturelles*, **1969**, *269*, 1334-1337.

130. Klancnik, A.; Piskernik, S.; Jersek, B.; Mozina, S.S. *Journal of microbiological methods*, **2010**, *81*, 121-126.

131. Gangadevi, J.M.V. *African Journal of Biotechnology*, **2007**, *6*, 1382-1386.

132. Karami, B.; Khodabakhshi, S.; Eskandari, K.; *Tet. Lett*. **2012**, *53*, 1445-1446.

133. Gjini, E.; Brito, P.H.; *PLoS computational biology,* **2016**, *12*, e1004857. doi: 10.1371/journal.pcbi.1004857.

134. Khalafi-Nezhad, A.; Soltani Rad, M.N.; Mohabatkar, H.; Asrari, Z.; Hemmateenejad, B. *Bioorg. Med. Chem.* **2005**, *13*, 1931-1938.

135. Kobayashi, M.; Kinjo, T.; Koseki, Y.; Bourne, C.R.; Barrow, W.W.; Aoki, S. *Journal of chemical information and modeling,* **2014**, *54*, 1242-1253.

136. Mandalari,G.; Bennett, R.N.; Bisignano, G.; Trombetta, D.; Saija, A.; Faulds, C.B.; Gasson, M.J.; Narbad, A. *Journal of applied microbiology,* **2007**, *103*, 2056-2064.

137. Miceli, N.; Trovato, A.; Dugo, P.; Cacciola, F.; Donato, P.; Marino, A.; Bellinghieri, V.; La Barbera, T.M.; Guvenc, A.; Taviano, M.F. *Journal of agricultural and food chemistry*, **2009**, *57*, 6570-6577.

138. Orhan, D.D.; Özçelik, B.; Özgen, S.; Ergun, F. *Microbiological Research,* **2010**, *165*, 496-504.

139. Cushnie, T.P.; Lamb, A.J. *International journal of antimicrobial agents*, **2005**, *26*, 343-356.

140. Ventura, T.L.; Calixto, S.D.; de Azevedo Abrahim-Vieira, B.; de Souza, A.M.; Mello, M.V.; Rodrigues, C.R.; Soter de Mariz e Miranda, L.; Alves de Souza, R.O.; Leal, I.C.; Lasunskaia, E.B.; Muzitano, M.F. *Molecules,* **2015**, *20*, 8072-8093.

141. Alcaraz, L.E.; Blanco, S.E.; Puig, O.N.; Tomas, F.; Ferretti, F.H. *Journal of theoretical biology,* **2000**, *205*, 231-240.

142. An, J.; Zuo, G.Y.; Hao, X.Y.; Wang, G.C.; Li, Z.S. *Phytomedicine: international journal of phytotherapy and phytopharmacology,* **2011**, *18*, 990-993.

143. Mercader, A.G.; Pomilio, A.P. *Anti-Infective Agents* **2012**, *10*, 41-54.

144. Fang, Y.; Lu, Y.; Zang, X.; Wu, T.; Qi, X.; Pan, S.; Xu, X. *Scientific Reports,* **2016***, 6*, 23634. doi: 10.1038/srep23634 http://www.nature.com/articles/srep23634#supplementary-information.

145. Forbes, A.M.; Lin, H.; Meadows, G.G.; Meier, G.P. *International journal of oncology*, **2014**, *45*, 831-842.

146. Agullo, G.; Gamet-Payrastre, L.; Manenti, S.; Viala, C.; Remesy, C.; Chap, H.; Payrastre, B. *Biochemical pharmacology*, **1997**, *53*, 1649-1657.

147. Haddad, A.Q.; Venkateswaran, V.; Viswanathan, L.; Teahan, S.J.; Fleshner, N.E.; Klotz, L.H. *Prostate cancer and prostatic diseases*, **2006**, *9*, 68-76.

148. Francisco, A. *Phytochemical Analysis* **1995**, *6*, 55-55.

149. Goto, H.; Terao, Y.; Akai, S. *Chemical & pharmaceutical bulletin*, **2009**, *57*, 346-360.

150. Woo, Y.; Shin, S.Y.; Hyun, J.; Lee, S.D.; Lee, Y.H.; Lim, Y. *International journal of molecular medicine*, **2012**, *29*, 403-408.

151. Frydoonfar, H.R.; McGrath, D.R.; Spigelman, A.D. *Colorectal disease: the official journal of the Association of Coloproctology of Great Britain and Ireland,* **2003**, *5*, 149-152.

152. Kretzschmar, G.; Vollmer, G.; Schwab, P.; Tischer, S.; Metz, P.; Zierau, O. *The Journal of steroid biochemistry and molecular biology*, **2007**, *107*, 114-119.

153. Szkudelska, K.; Nogowski, L.; Nowicka, E.; Szkudelski, T. *Journal of animal physiology and animal nutrition*, **2007**, *91*, 91-99.

154. Lee, S.; Shin, S.Y.; Lee, Y.; Park, Y.; Kim, B.G.; Ahn, J.H.; Chong, Y.; Lee, Y.H.; Lim, Y. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3866-3870.

155. Fang, Y.; Lu, Y.; Zang, X.; Wu, T.; Qi, X.; Pan, S.; Xu, X. *Sci. Rep.* **2016**, *6*, 23634; doi: 10.10 38/srep23634 (2016).

156. Martin, C.N.; Kennelly, J.C. *Drug. Metab. Rev.* **1985**, *16*, 89.

157. Collier, S.W.; Storm, J.E.; Bronaugh, R.L. *Toxicol. Appl. Pharmacol.* **1993**, *118*, 73.

158. Levine, W.G. *Drug. Metab. Rev.* **1991**, *23*, 253.

Appendix

A.1 NMR data for Chapter two

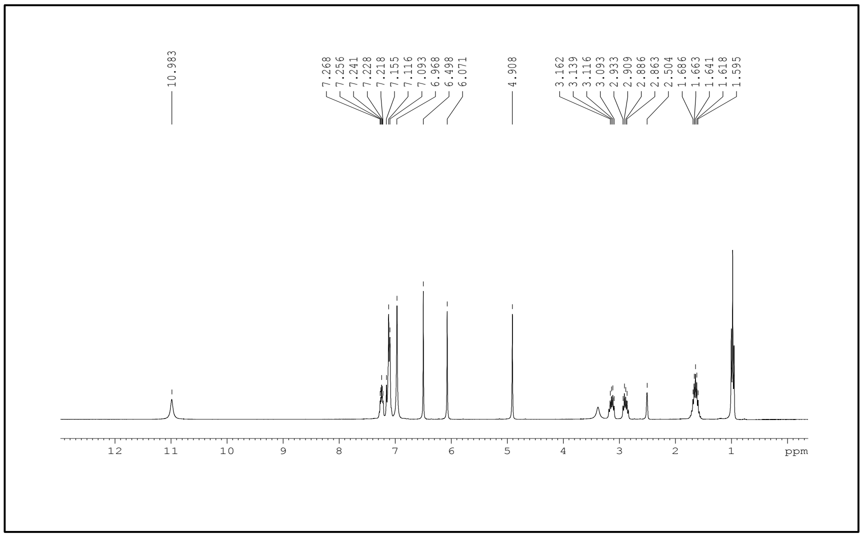


Figure A.1: ^{1}H NMR spectrum of **2.2a**

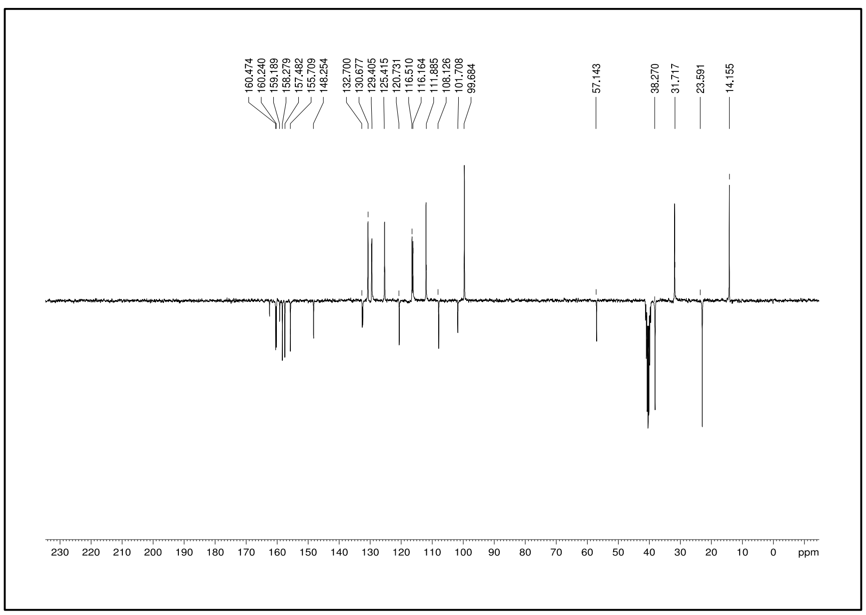


Figure A.2: DEPTQ135 ^{13}C NMR spectrum of **2.2a**

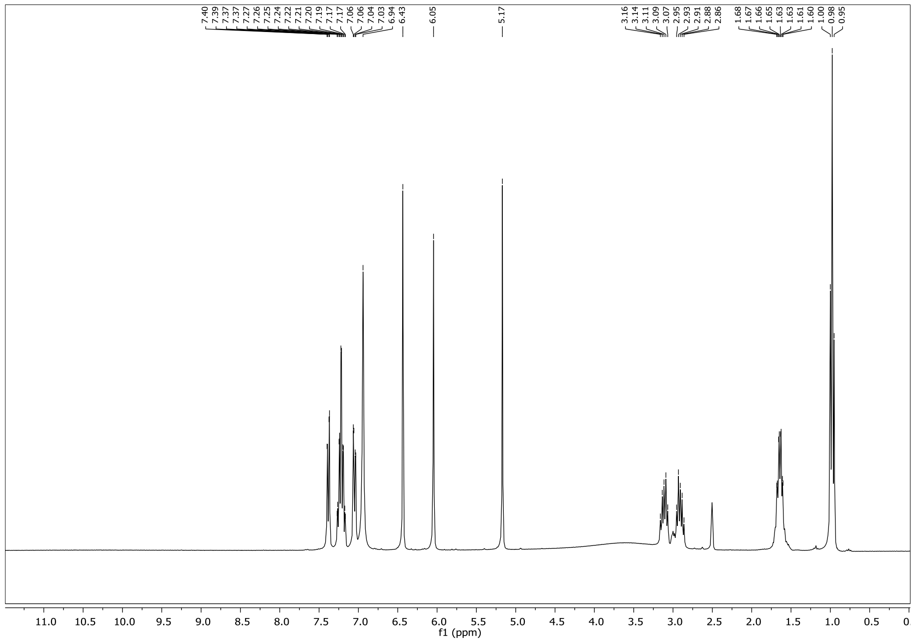


Figure A.3: ^{1}H NMR spectrum of **2.2b**

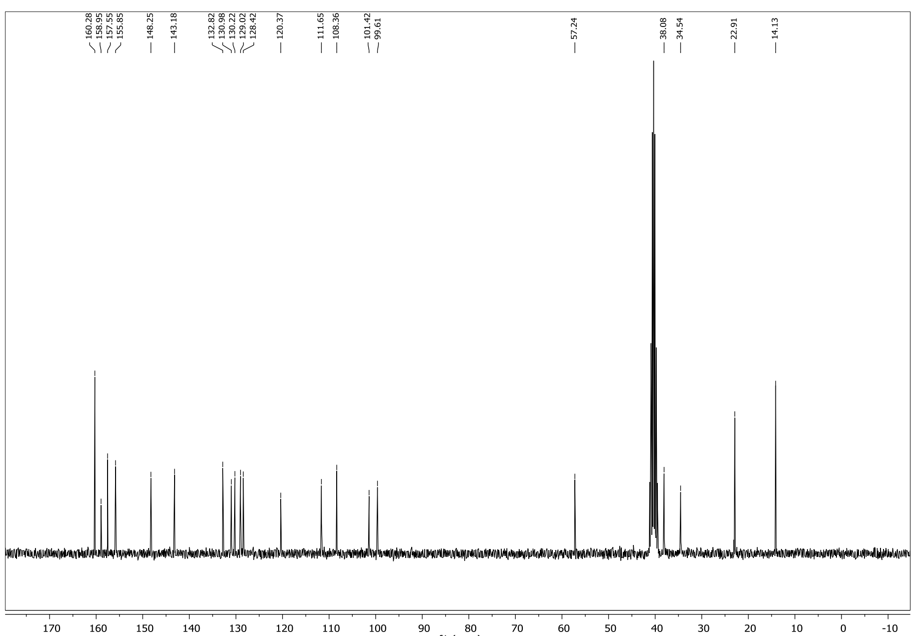


Figure A.4: DEPTQ135 ^{13}C NMR spectrum of **2.2b**

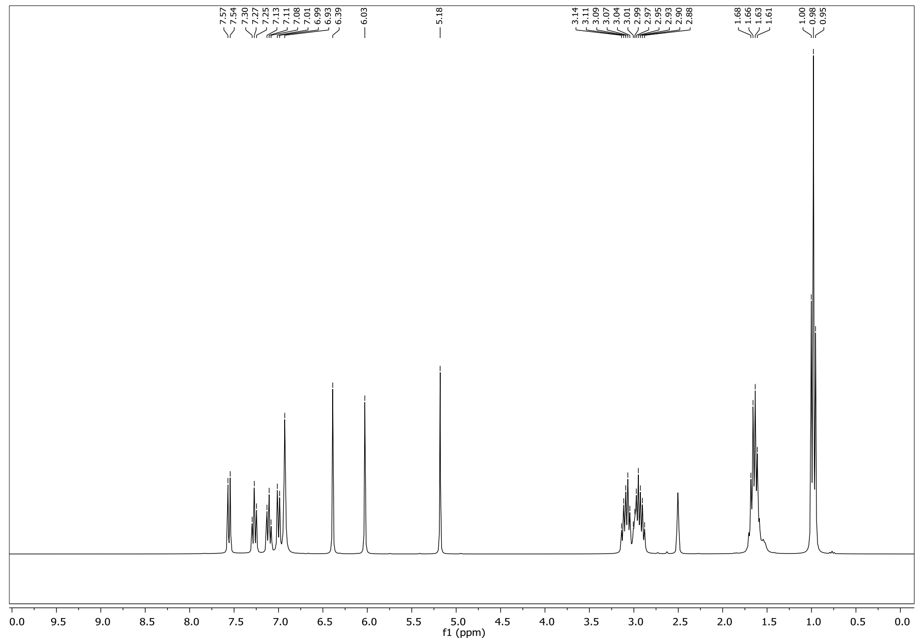


Figure A.5: ^{1}H NMR spectrum of **2.2c**

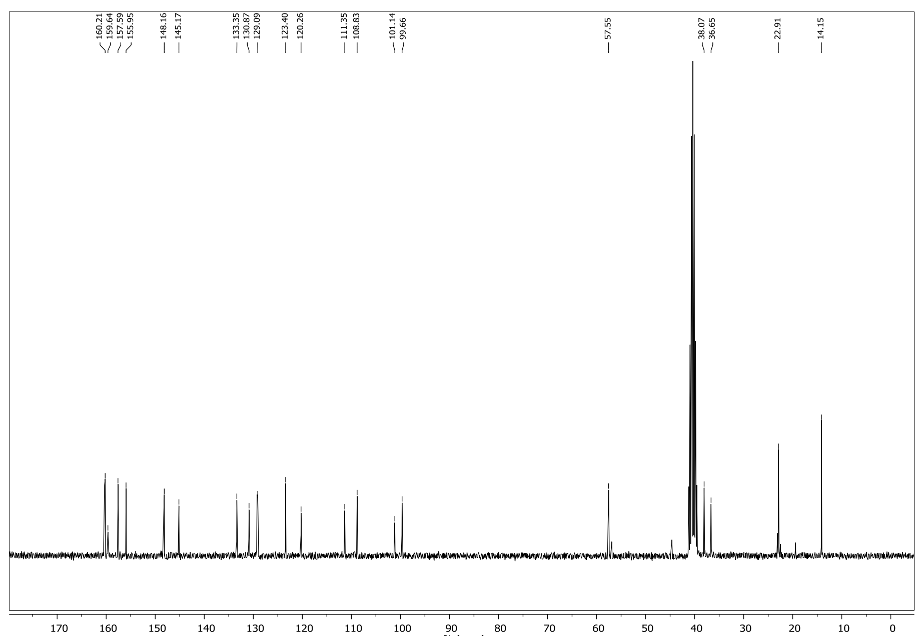


Figure A.6: ^{13}C NMR spectrum of **2.2c**

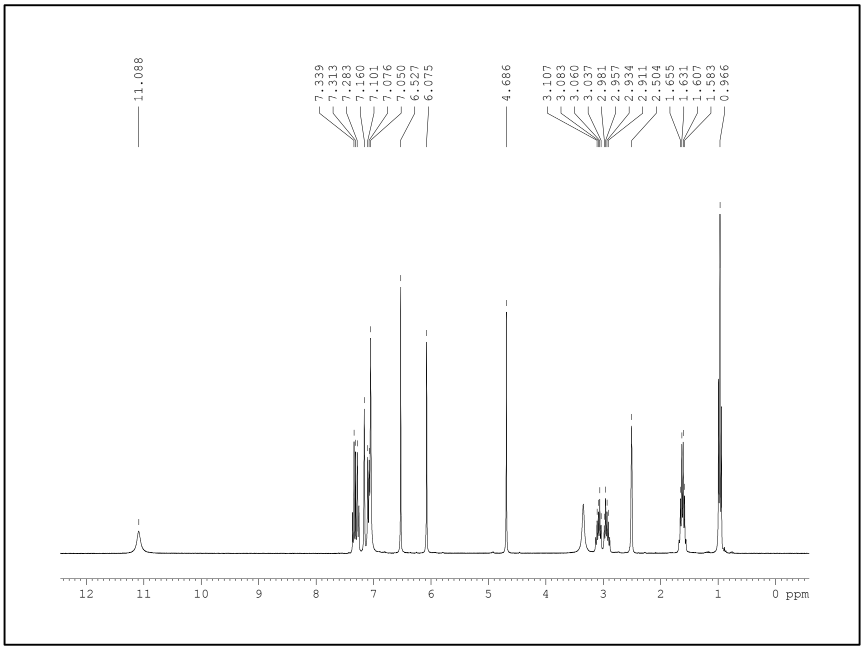


Figure A.7: ^{1}H NMR spectrum of **2.2d**

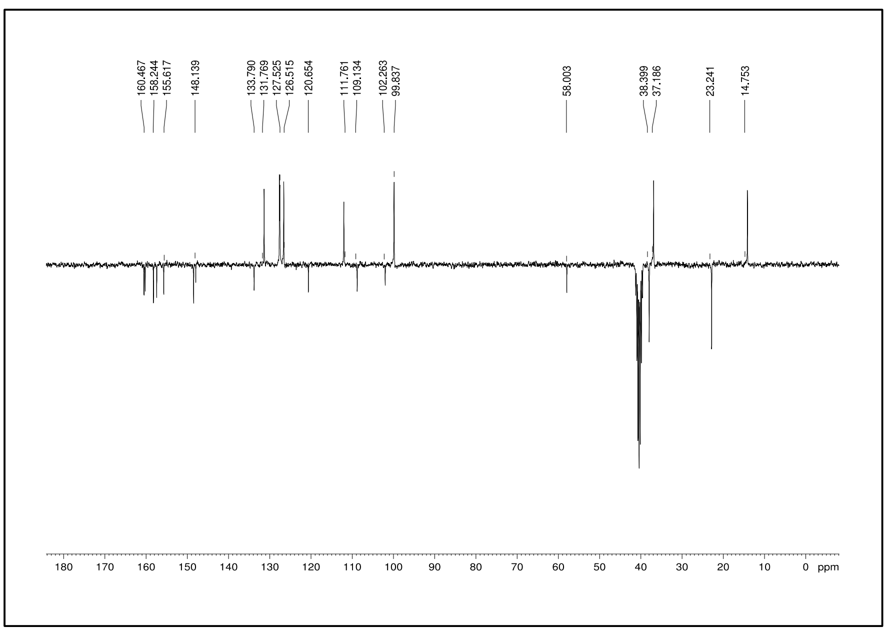


Figure A.8: DEPTQ135 ^{13}C NMR spectrum of **2.2d**

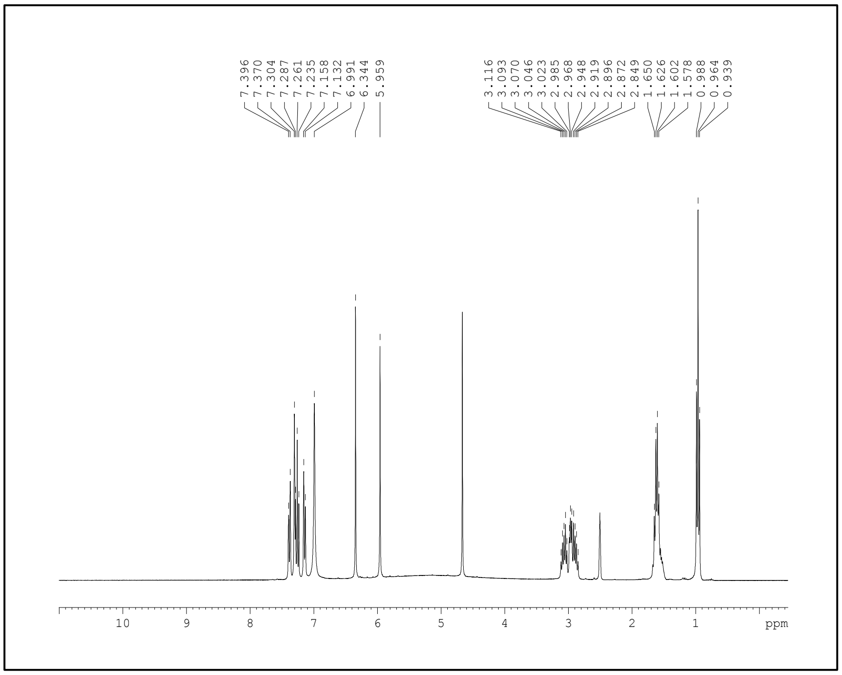


Figure A.9: ^{1}H NMR spectrum of **2.2e**

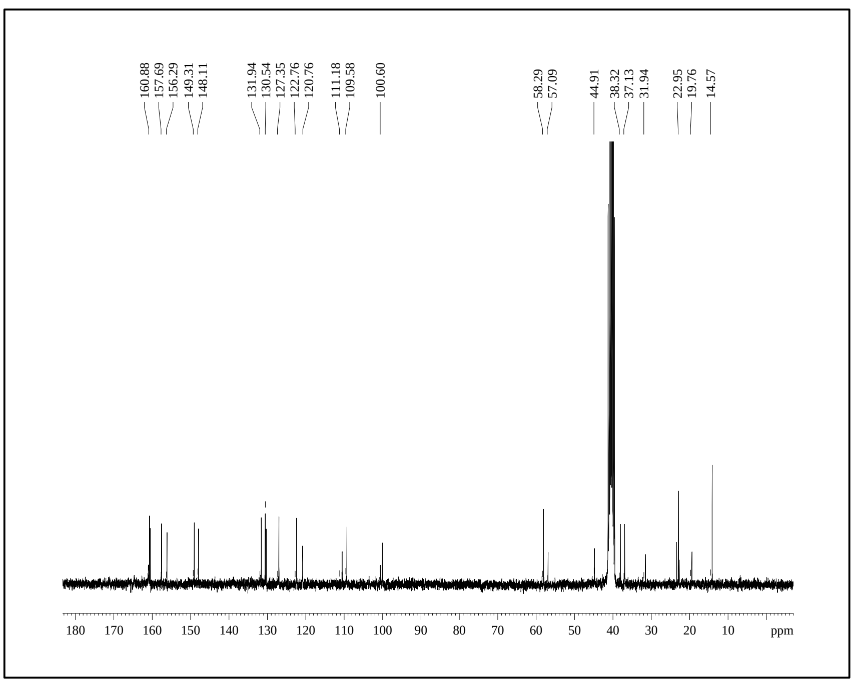


Figure A.10: ^{13}C NMR spectrum of **2.2e**

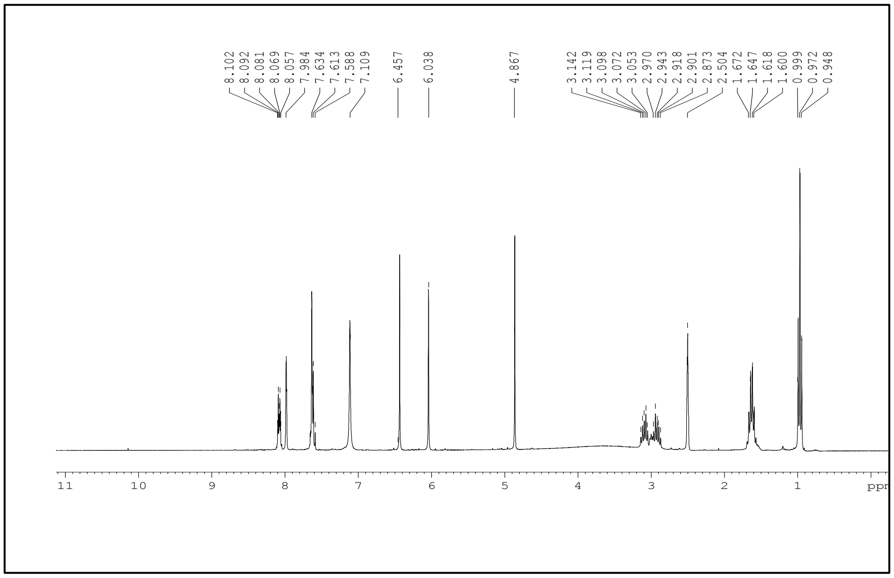


Figure A.11: ^{1}H NMR spectrum of **2.2f**

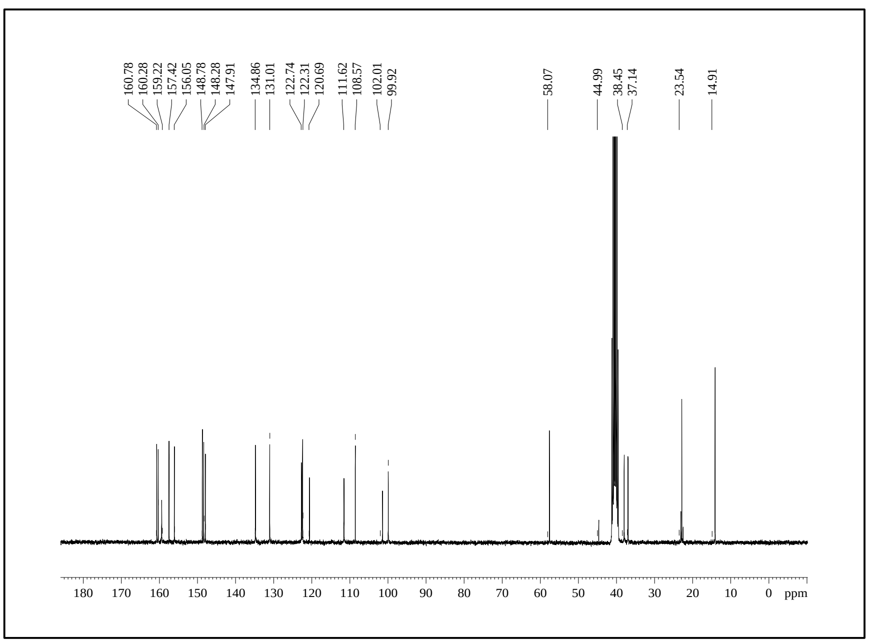


Figure A.12: ^{13}C NMR spectrum of **2.2f**

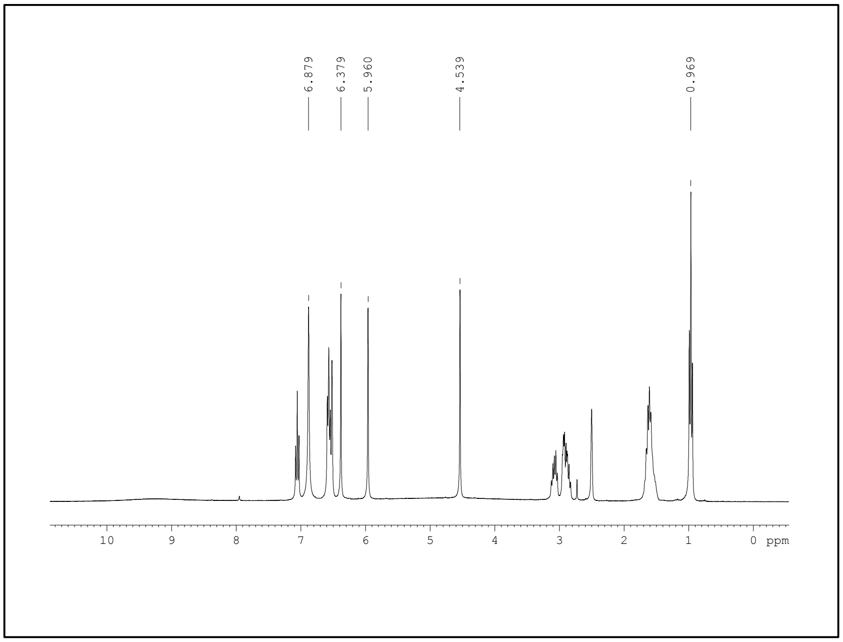


Figure A.13: ^{1}H NMR spectrum of **2.2g**

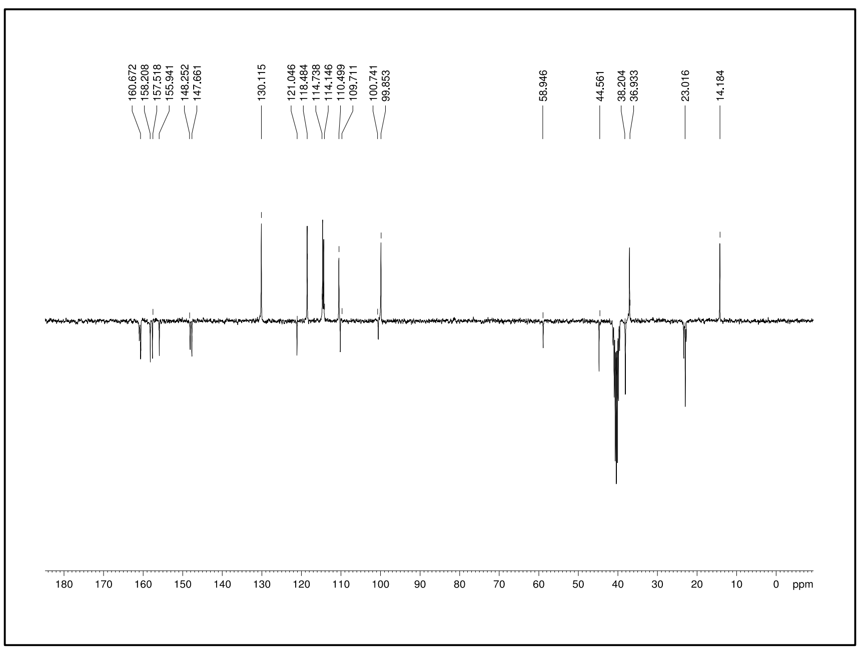


Figure A.14: DEPTQ135 ^{13}C NMR spectrum of **2.2g**

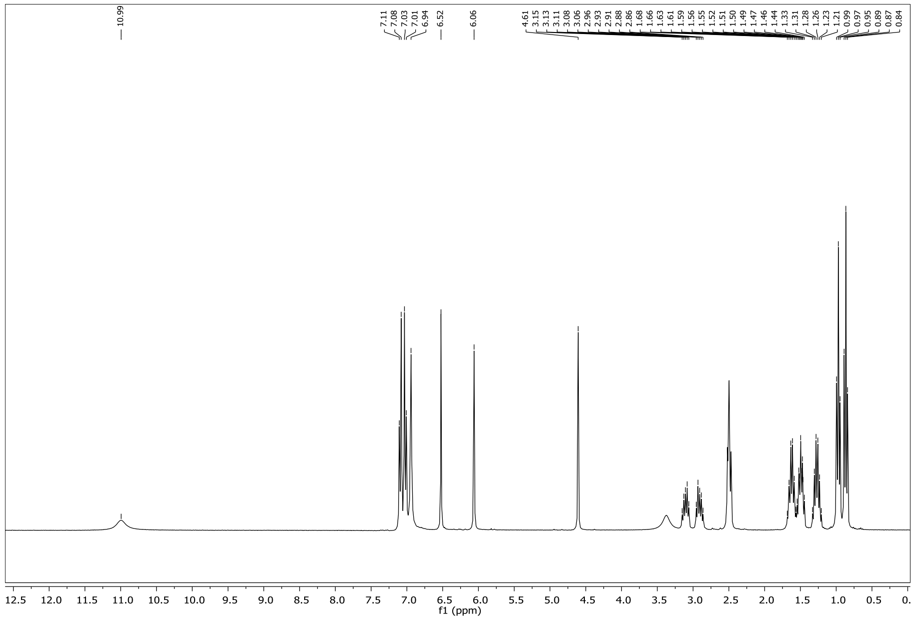


Figure A.15: ^{1}H NMR spectrum of **2.2h**

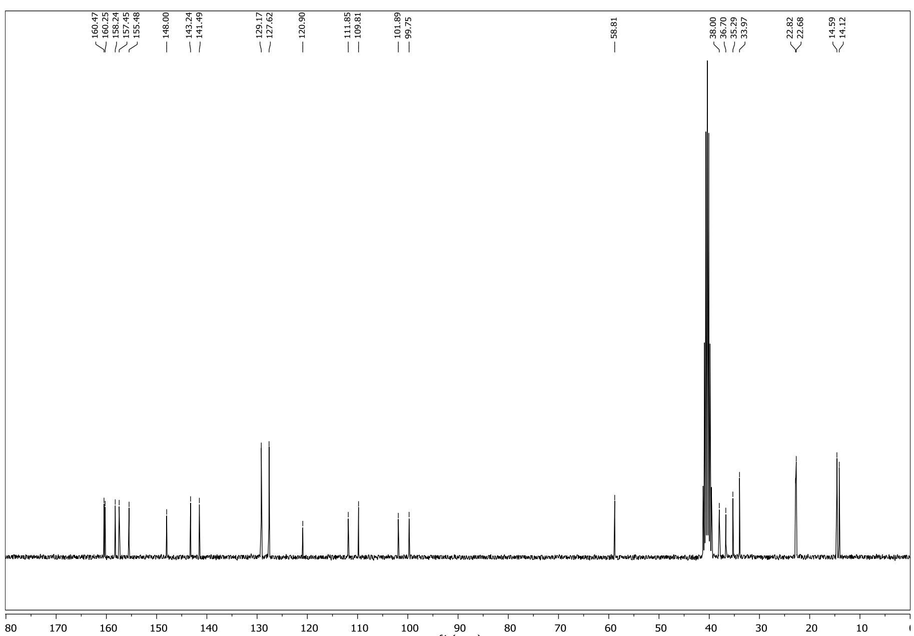


Figure A.16: ^{13}C NMR spectrum of **2.2h**

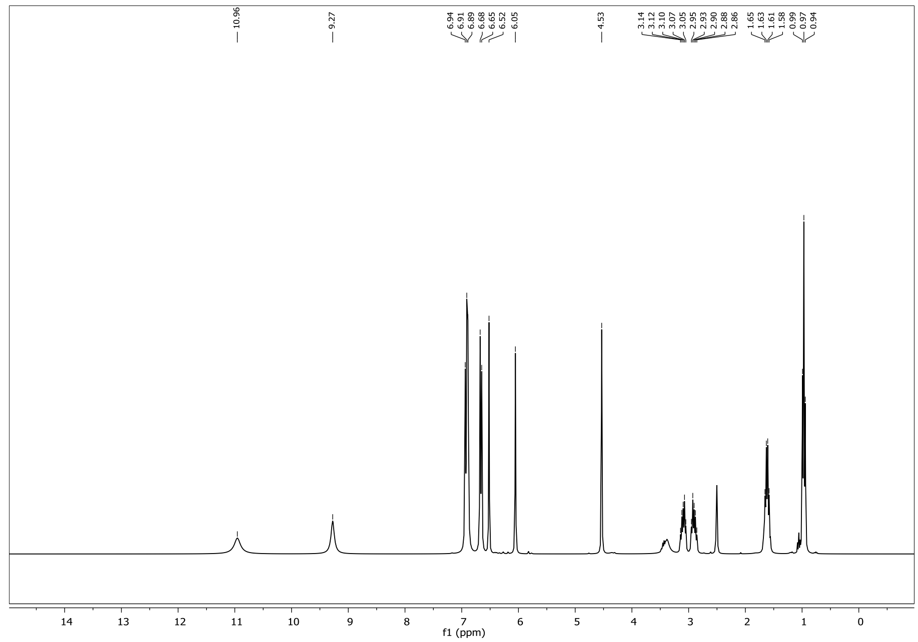


Figure A.17: ^{1}H NMR spectrum of **2.2i**

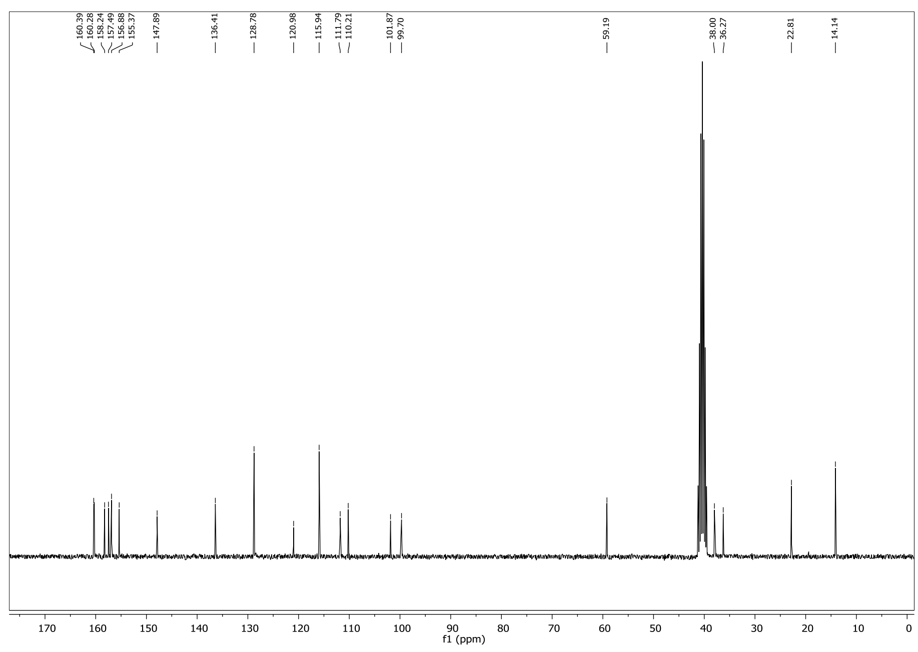


Figure A.18: ^{13}C NMR spectrum of **2.2i**

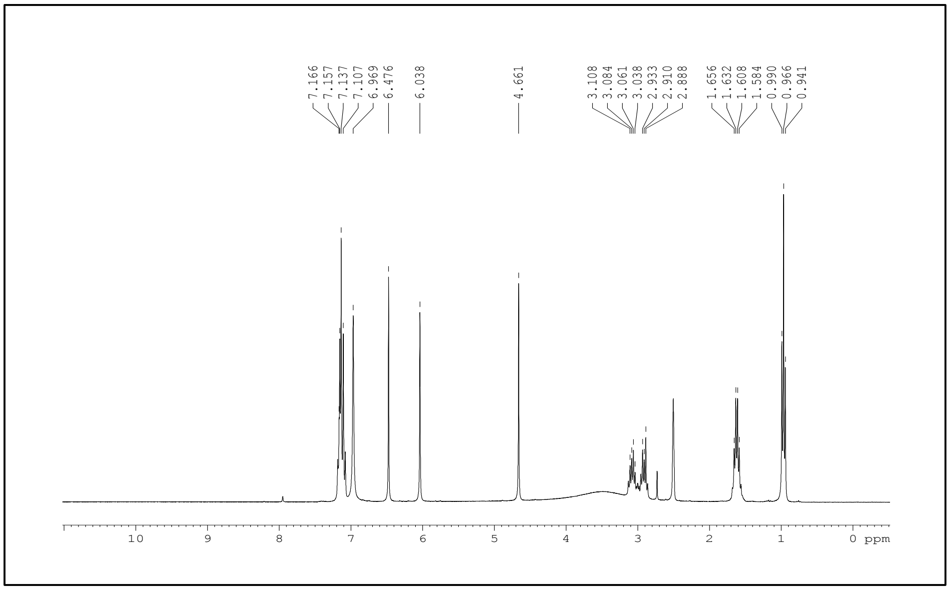


Figure A.19: ^{1}H NMR spectrum of **2.2j**

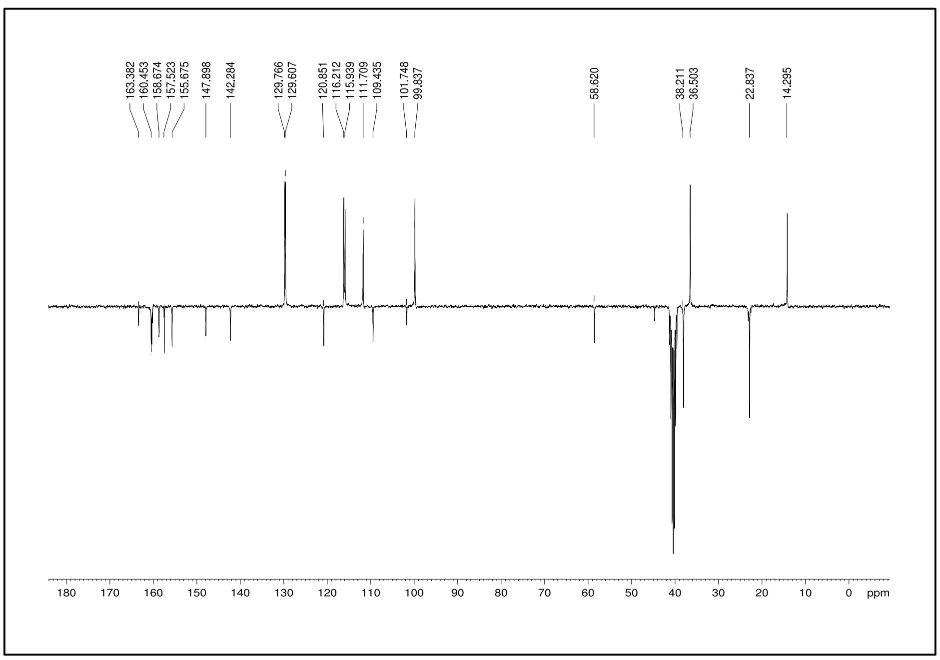


Figure A.20: DEPTQ135 ^{13}C NMR spectrum of **2.2j**

A.2 NMR data for Chapter Three

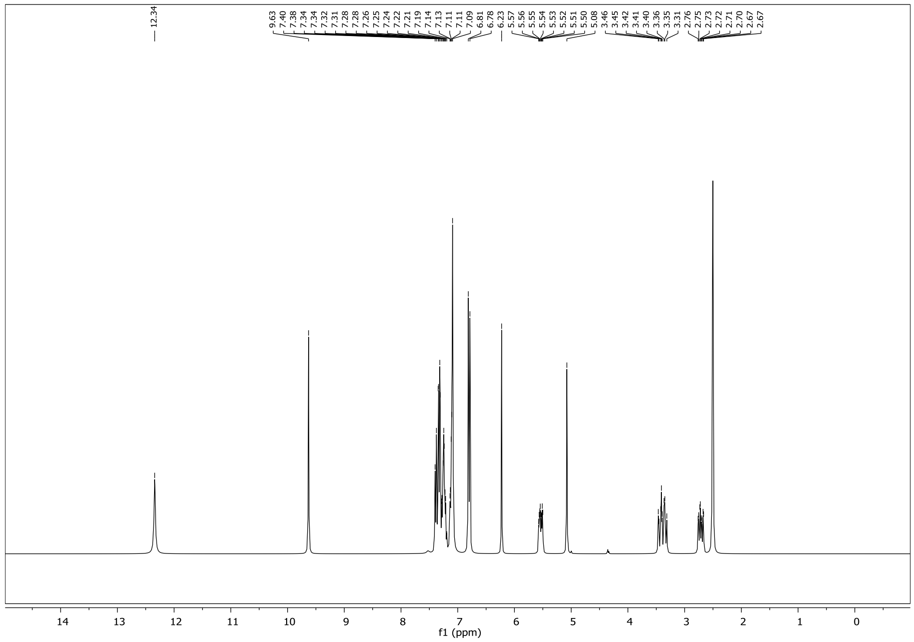


Figure A.21: ^{1}H NMR spectrum of **3.2a**

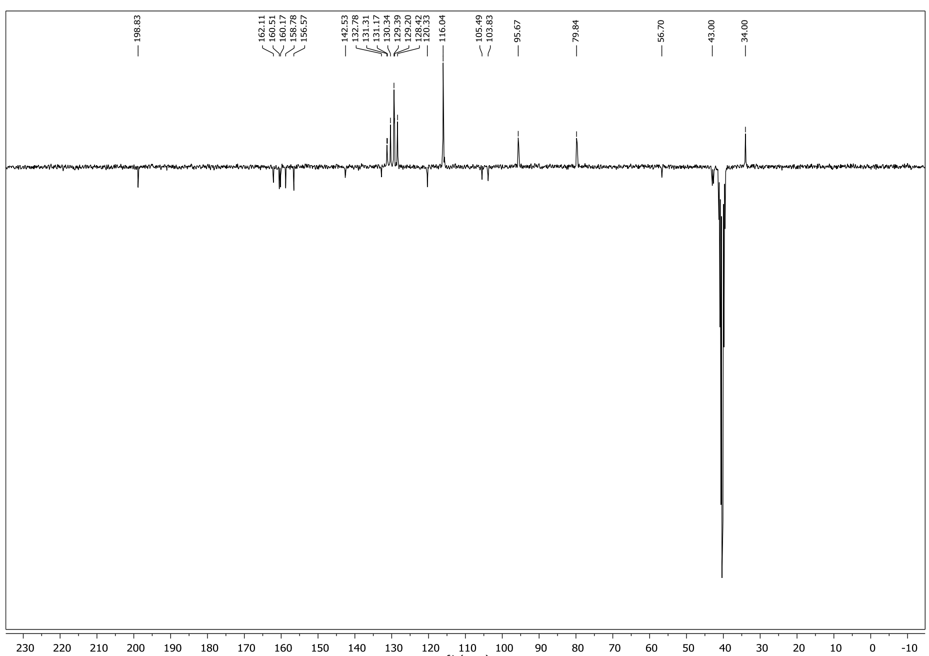


Figure A.22: DEPTQ135 ^{13}C NMR spectrum of **3.2a**

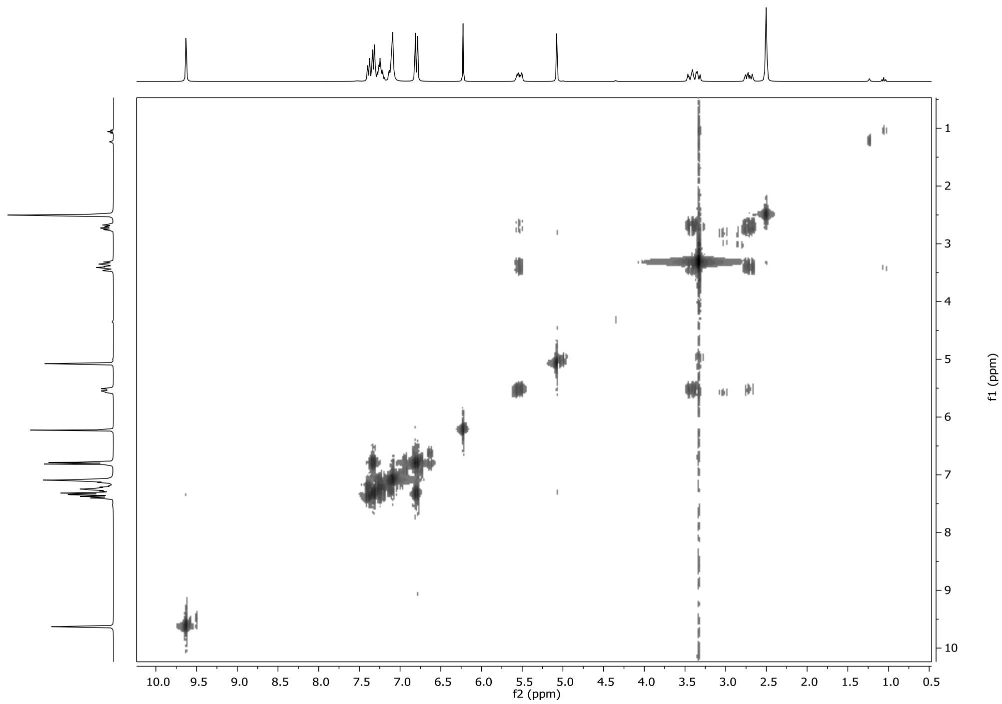


Figure A.23: COSY NMR spectrum of **3.2a**

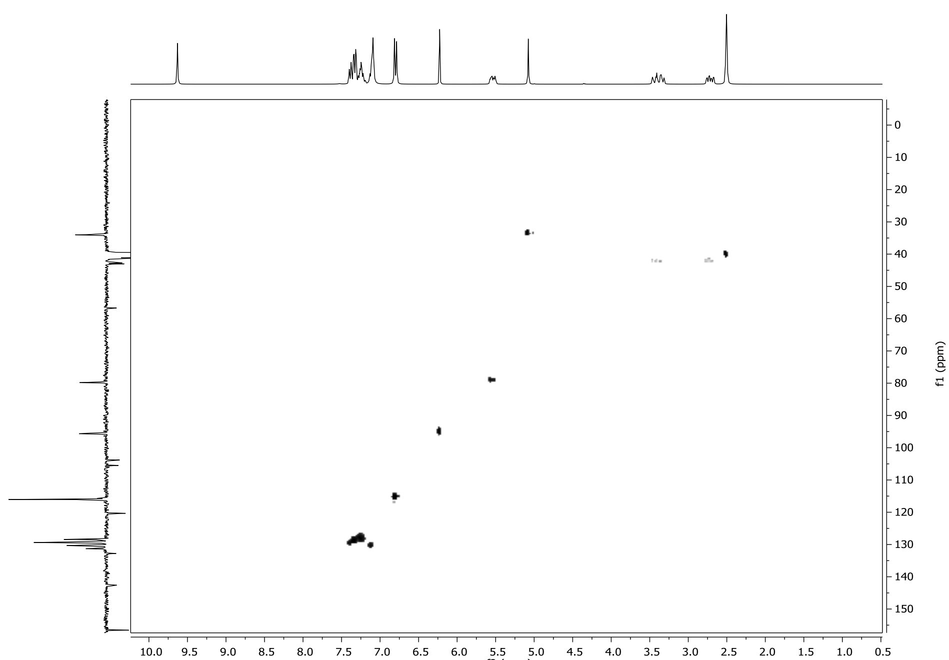


Figure A.24: HSQC NMR spectrum of **3.2a**

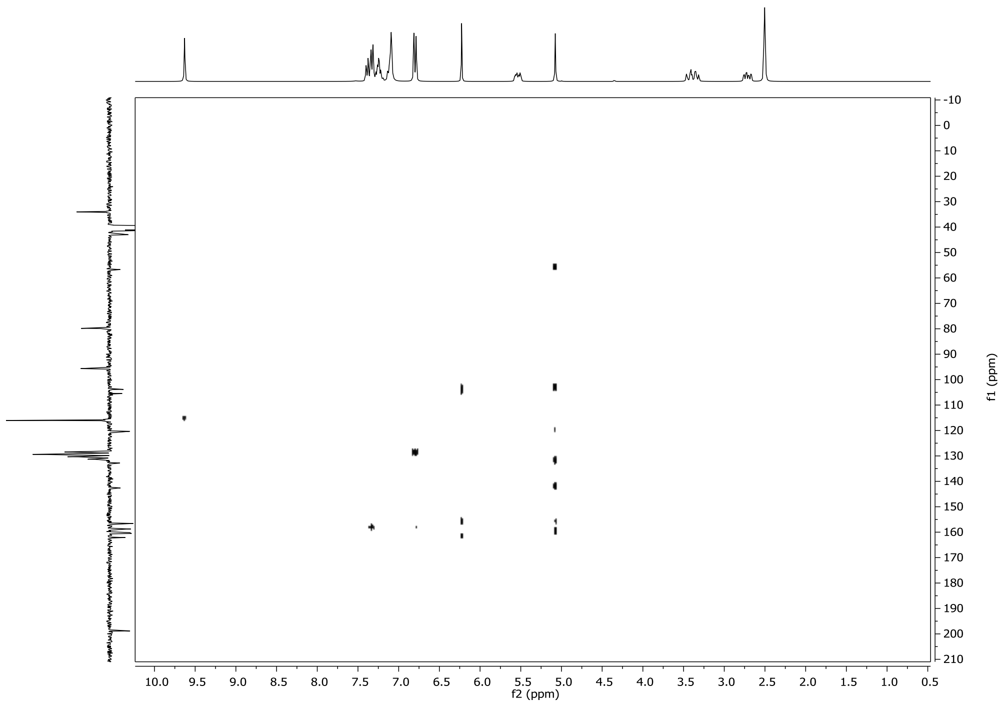


Figure A.25: HMBC NMR spectrum of **3.2a**

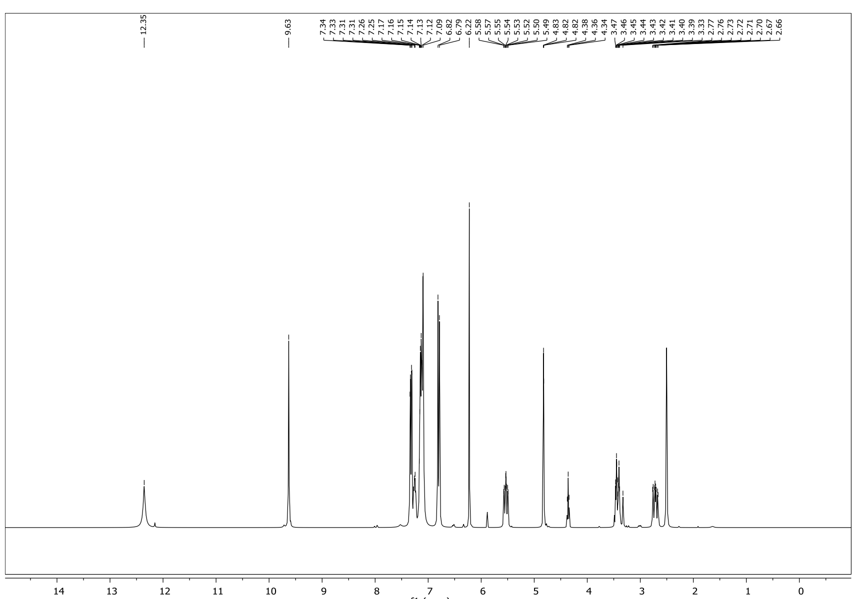


Figure A.26: ^{1}H NMR spectrum of **3.2b**

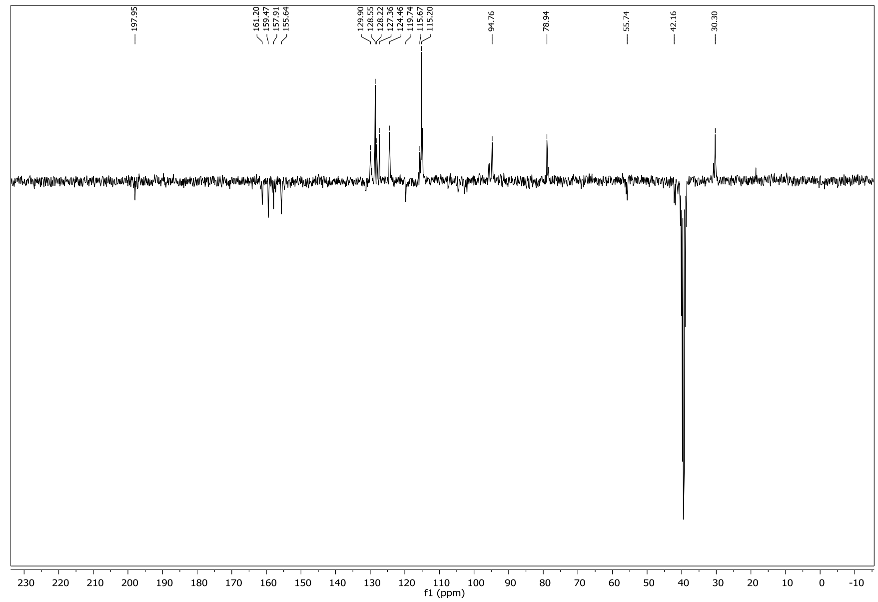


Figure A.27: DEPTQ135 ^{13}C NMR spectrum of **3.2b**

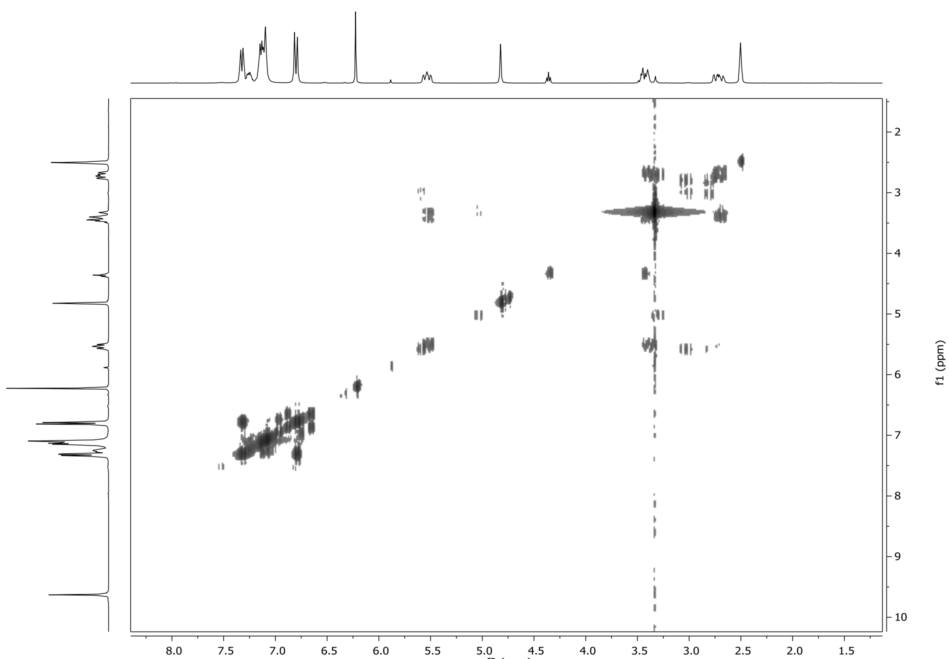


Figure A.28: COSY NMR spectrum of **3.2b**

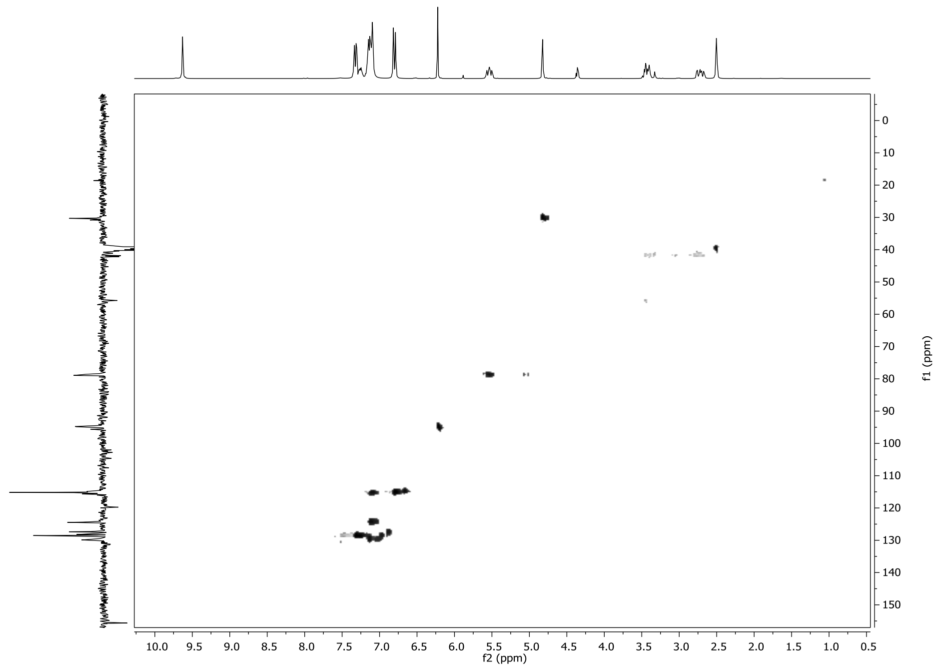


Figure A.29: HSQC NMR spectrum of **3.2b**

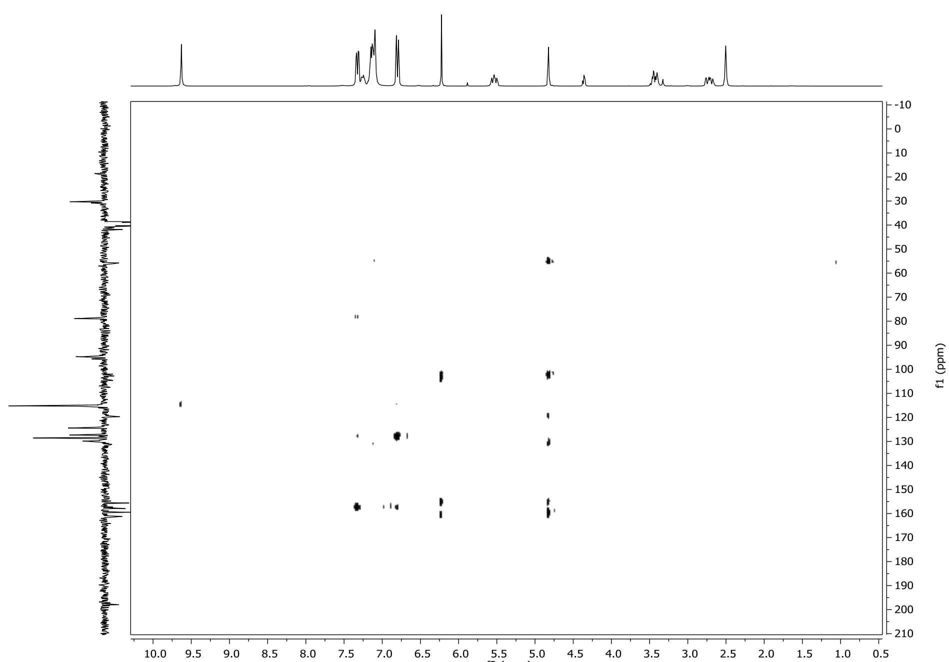


Figure A.30: HMBC NMR spectrum of **3.2b**

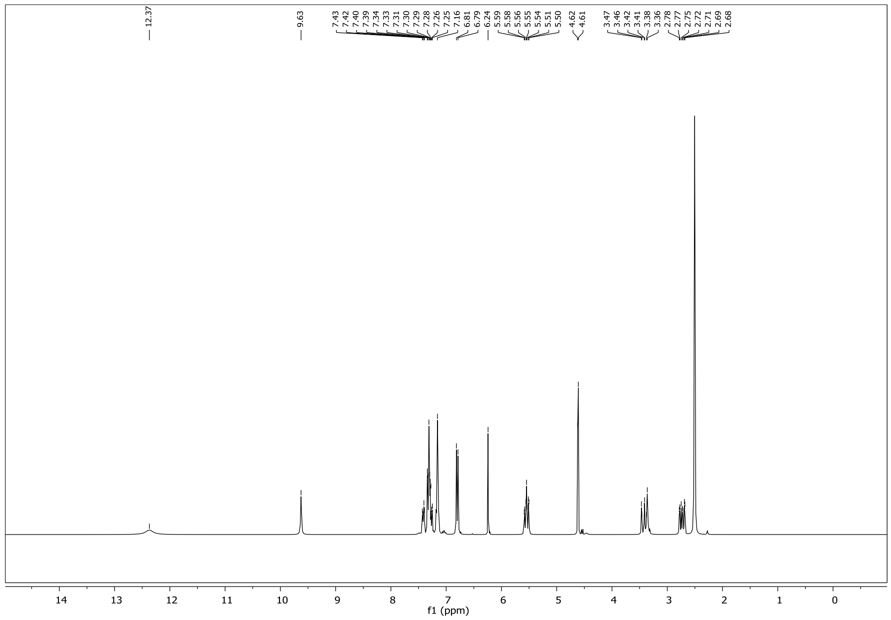


Figure A.31: ^{1}H NMR spectrum of **3.2c**

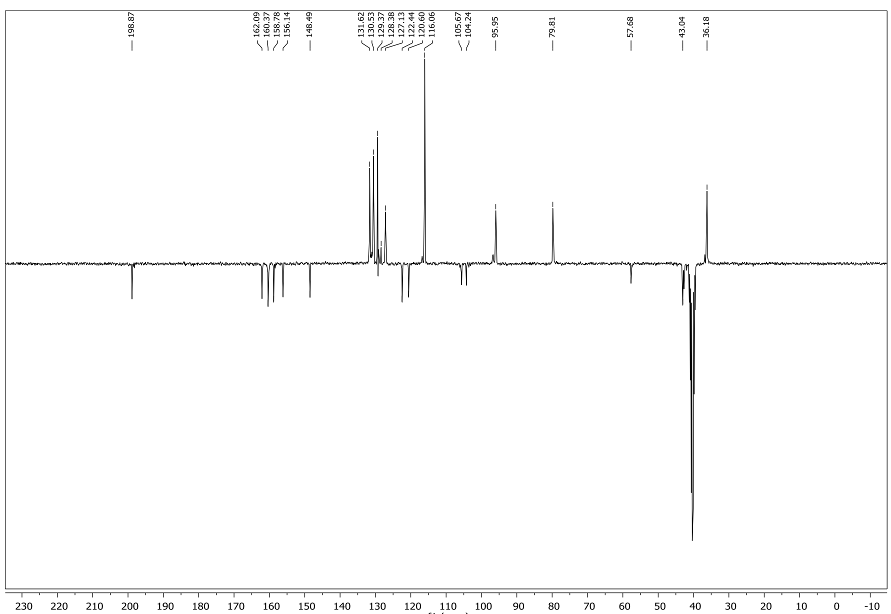


Figure A.32: DEPTQ135 ^{13}C NMR spectrum of **3.2c**

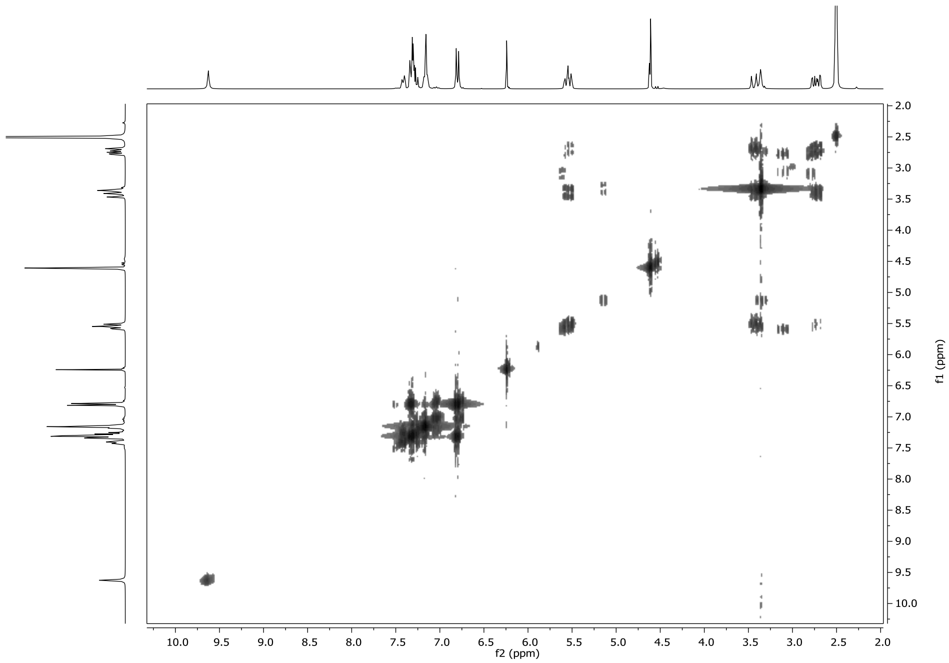


Figure A.33: COSY NMR spectrum of **3.2c**

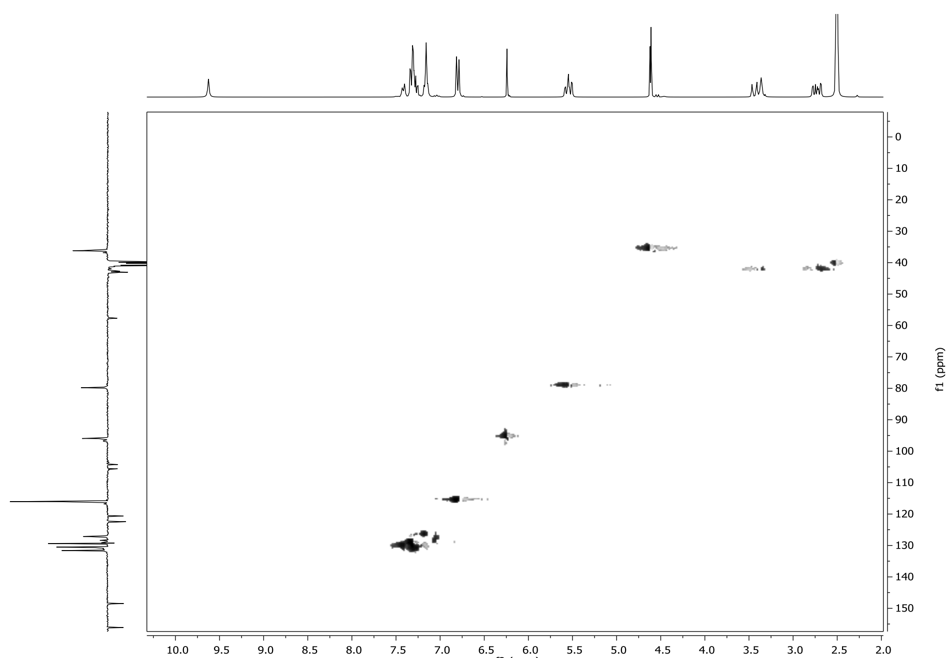


Figure A.34: HSQC NMR spectrum of **3.2c**

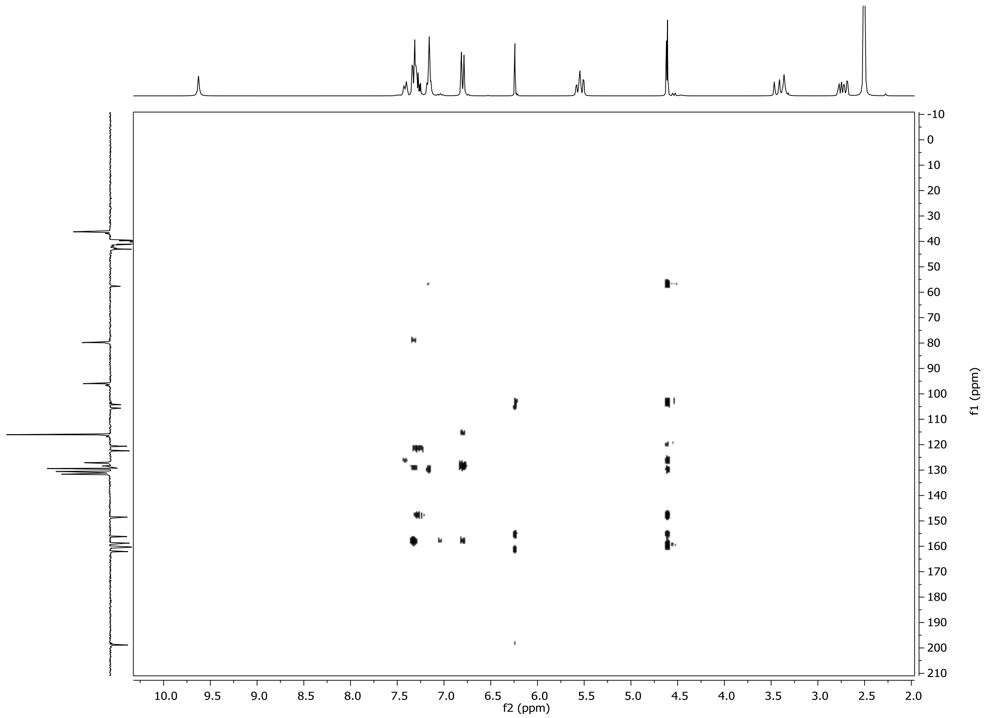


Figure A.35: HMBC NMR spectrum of **3.2c**

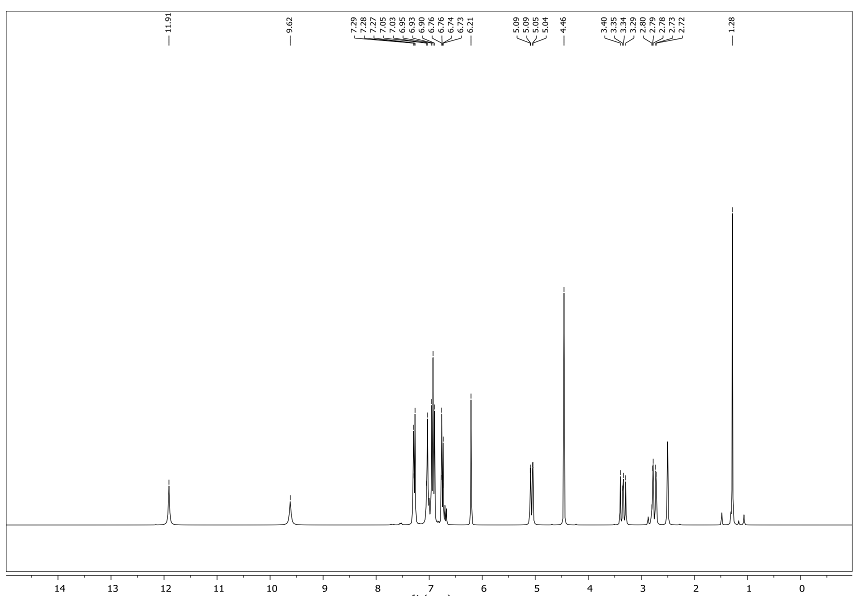


Figure A.36: ^{1}H NMR spectrum of **3.2d**

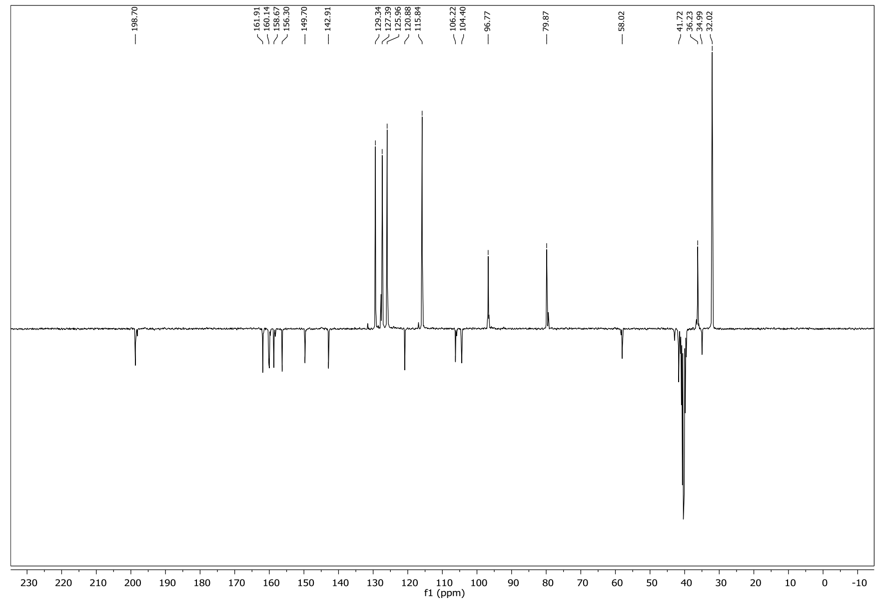


Figure A.37: DEPTQ135 ^{13}C NMR spectrum of **3.2d**

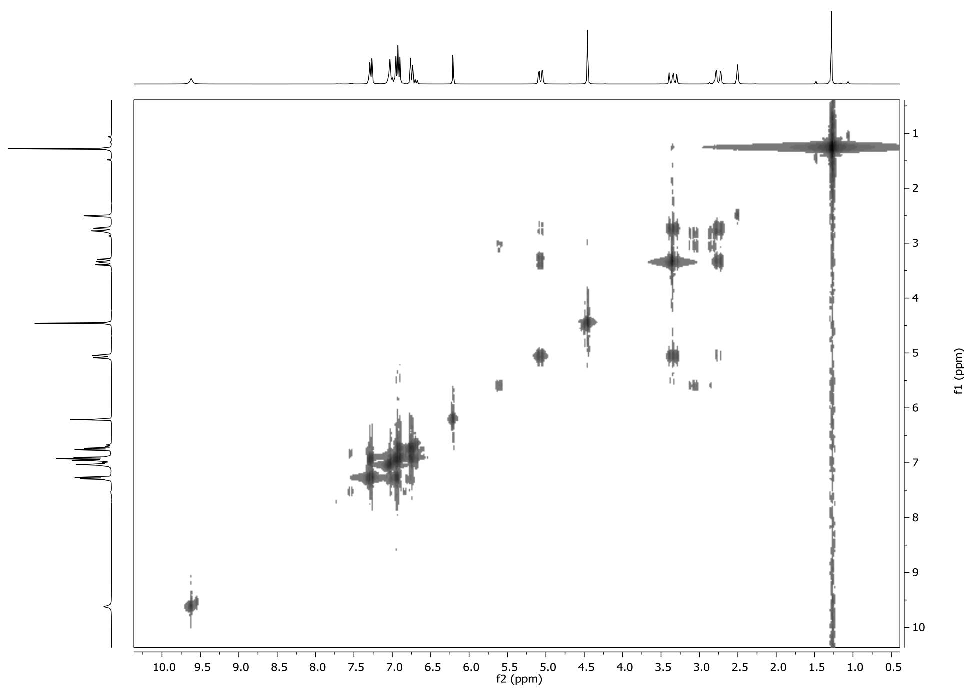


Figure A.38: COSY NMR spectrum of **3.2d**

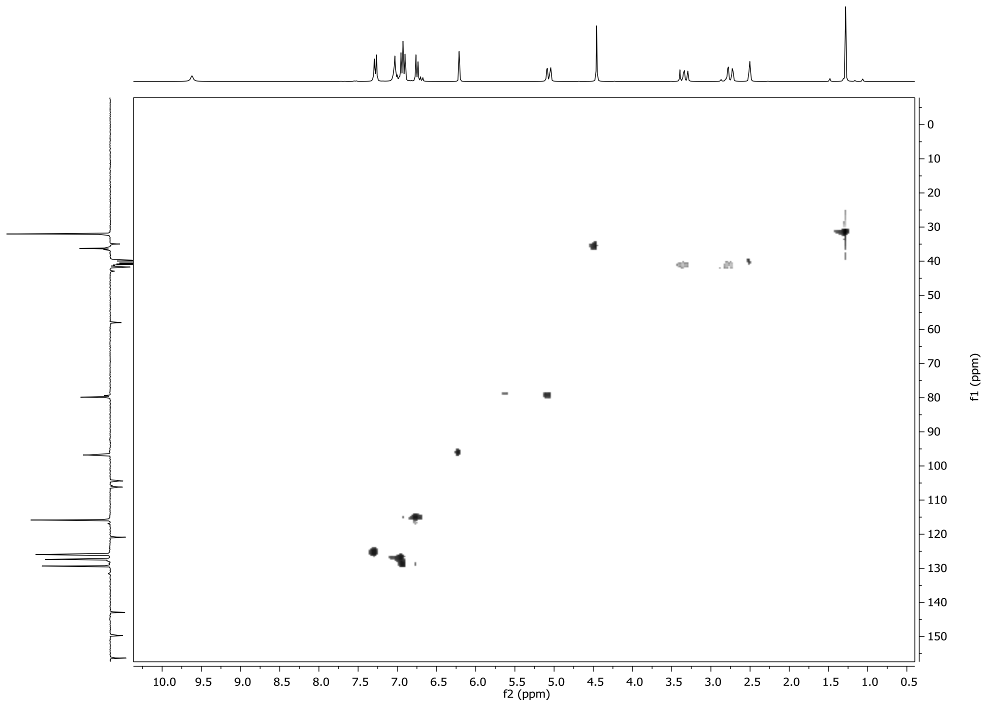


Figure A.39: HSQCNMR spectrum of **3.2d**

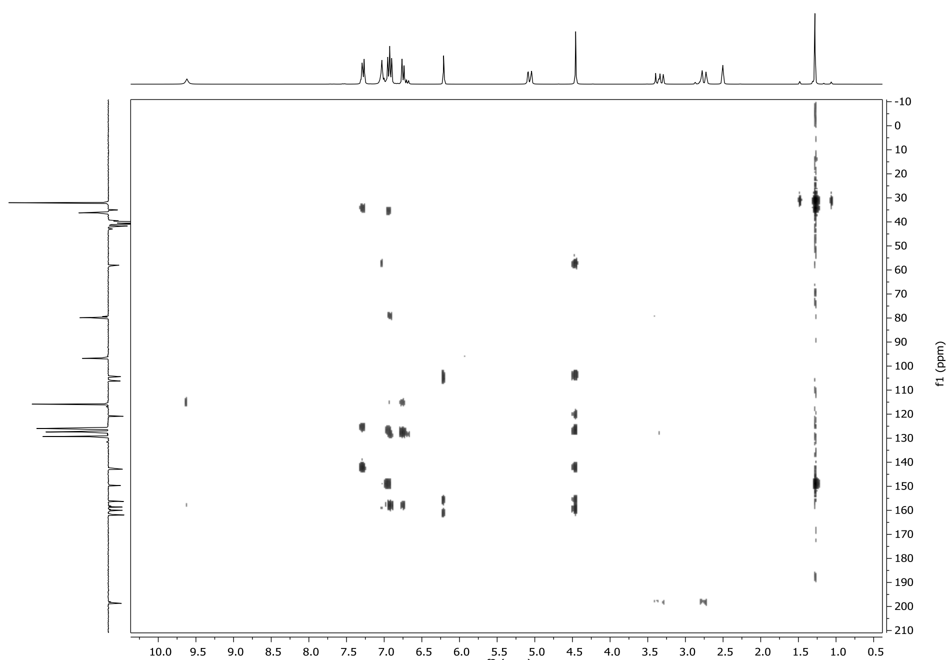


Figure A.40: HMBC NMR spectrum of **3.2d**

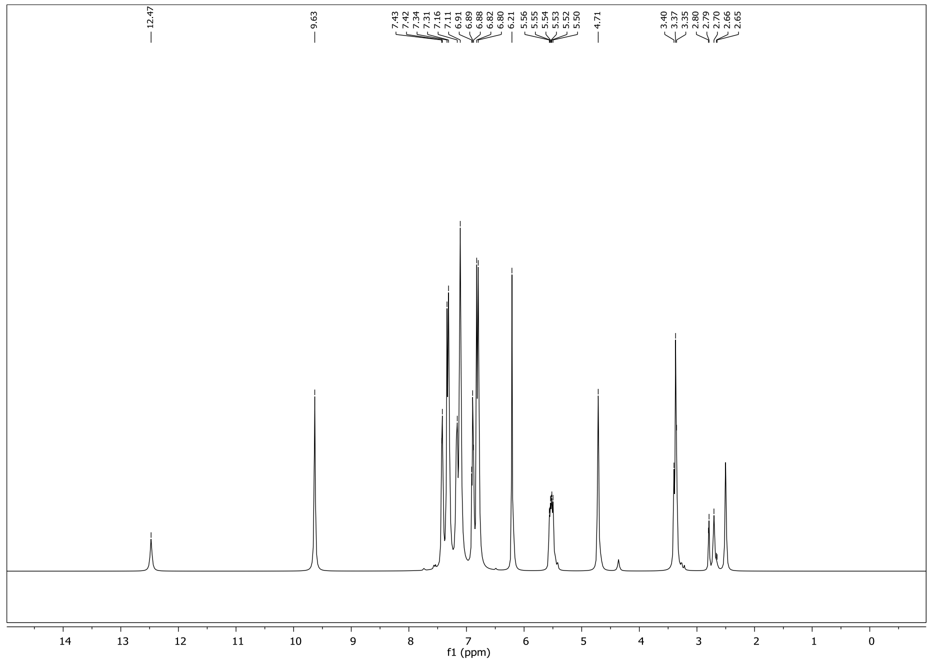


Figure A.41: ^{1}H NMR spectrum of **3.2e**

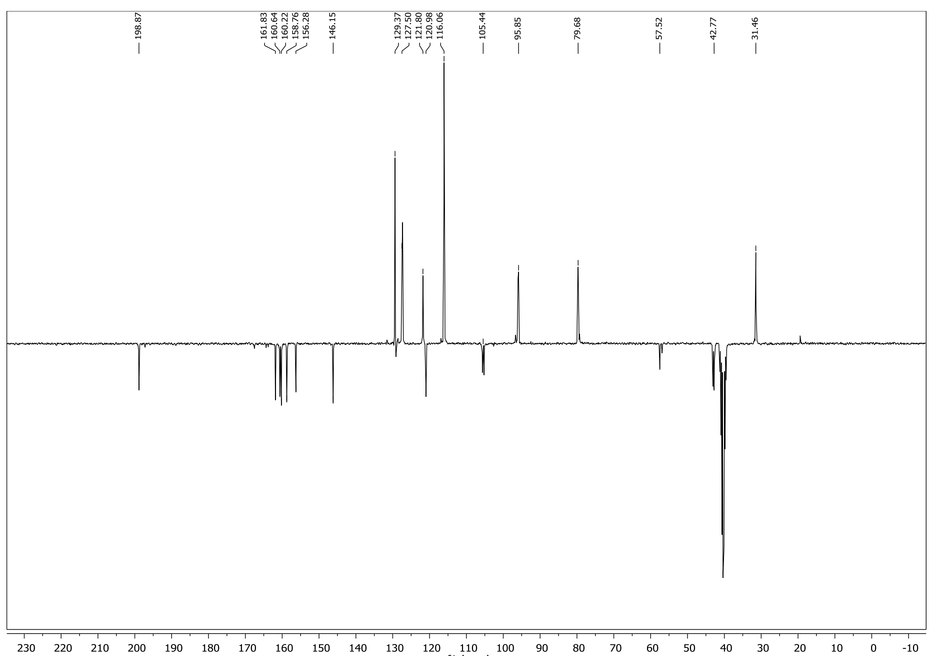


Figure A.42: DEPTQ135 ^{13}C NMR spectrum of **3.2e**

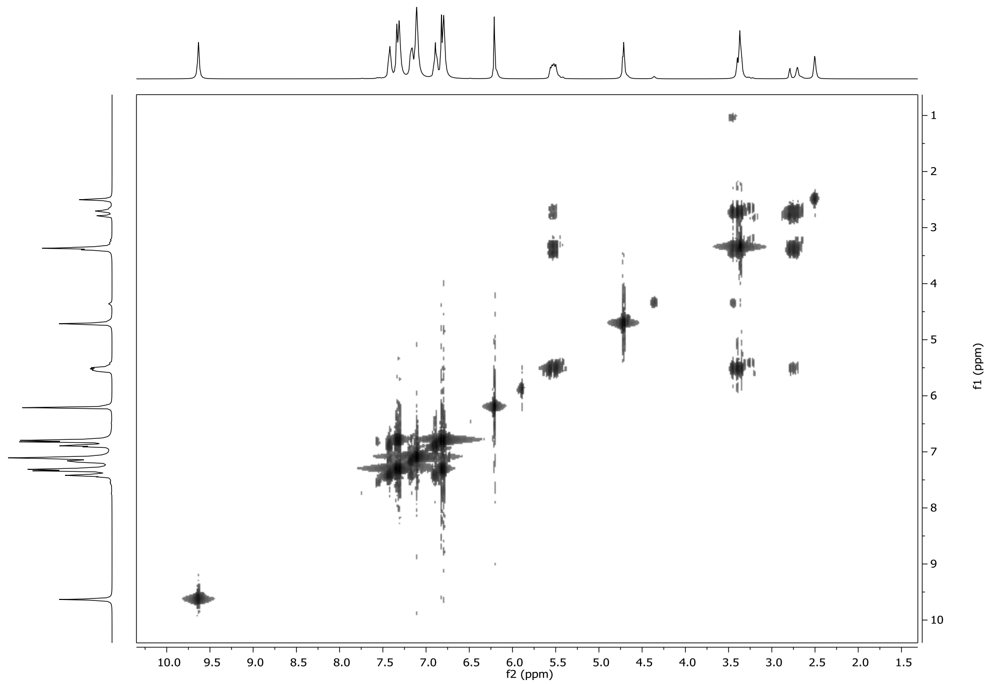


Figure A.43: COSY NMR spectrum of **3.2e**

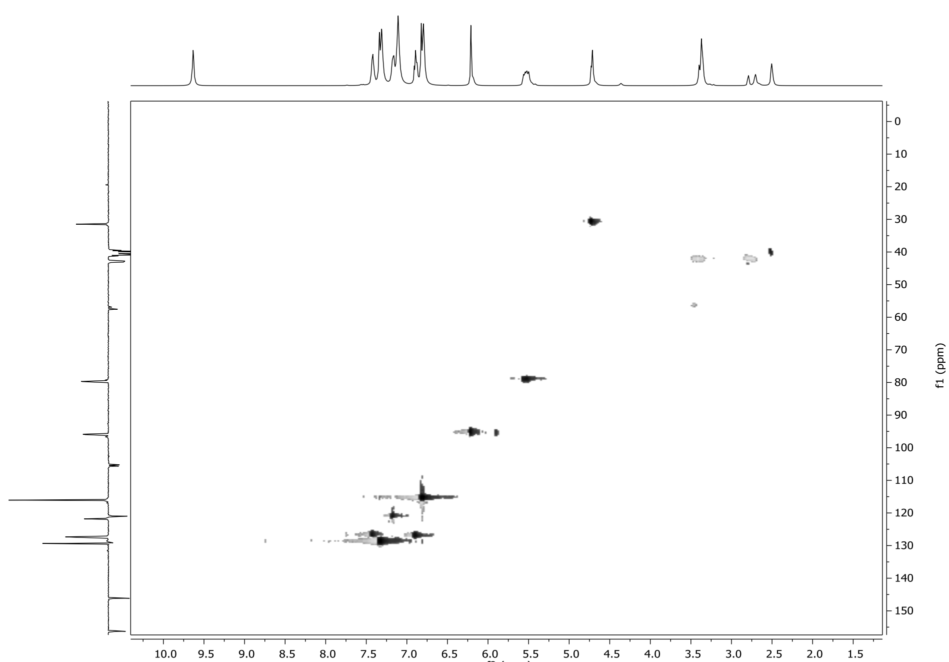


Figure A.44: HSQC NMR spectrum of **3.2e**

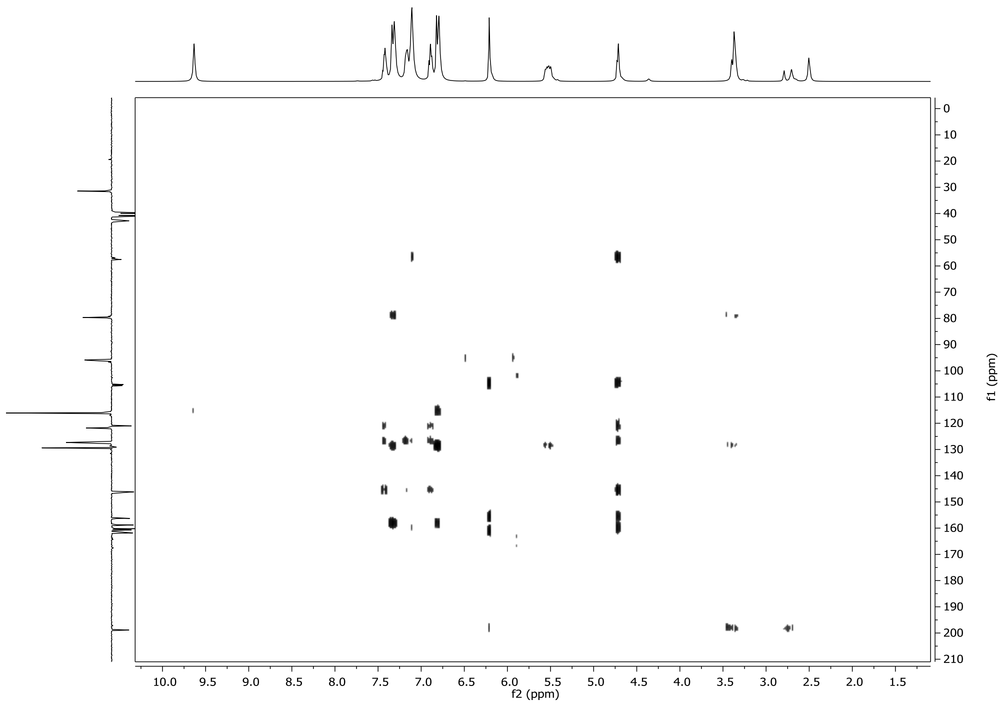


Figure A.45: HMBC NMR spectrum of **3.2e**

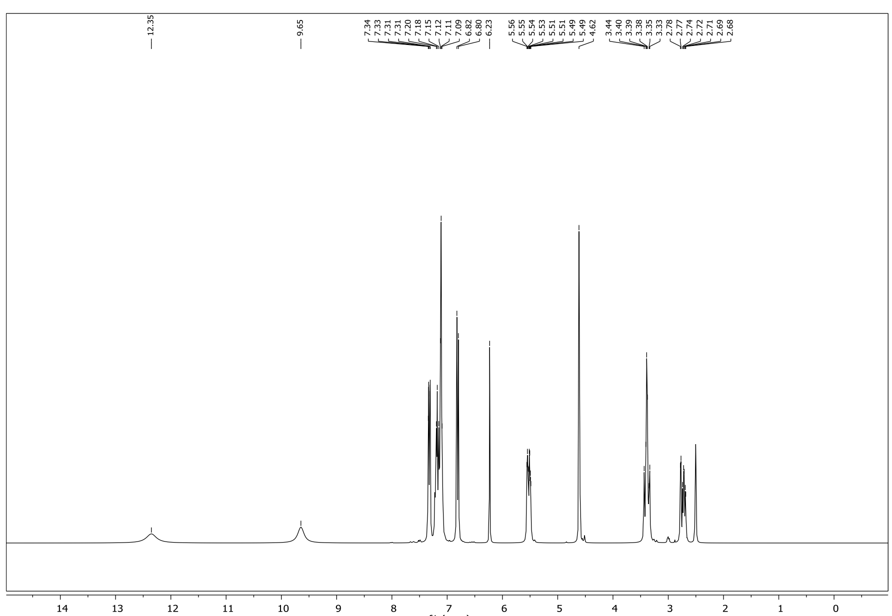


Figure A.46: ^{1}H NMR spectrum of **3.2f**

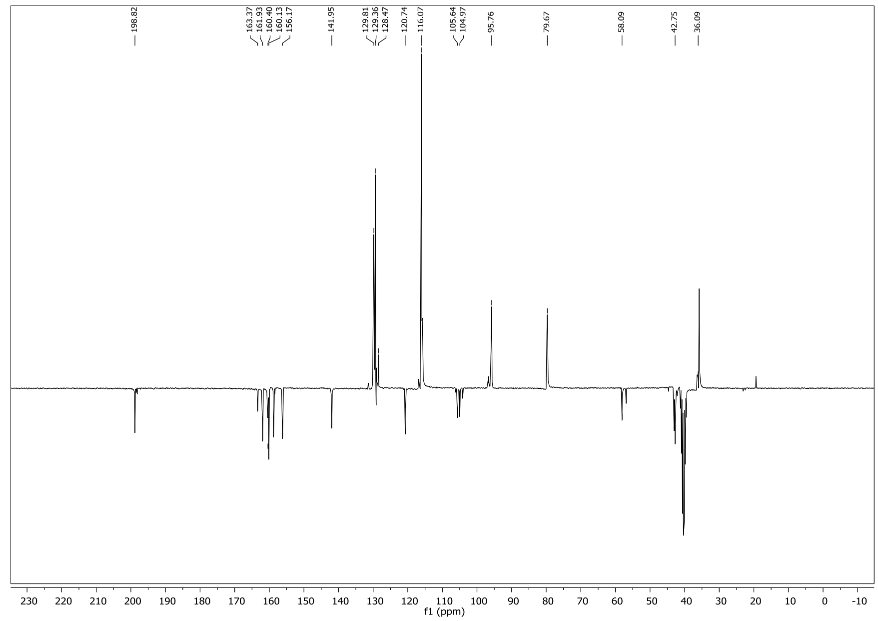


Figure A.47: DEPTQ135 ^{13}C NMR spectrum of **3.2f**

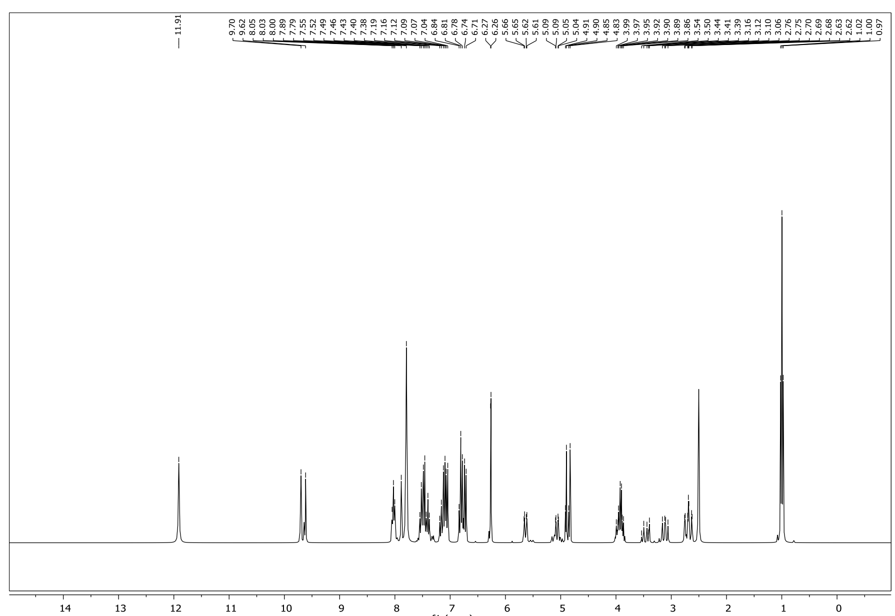


Figure A.48: ^{1}H NMR spectrum of **3.2g**

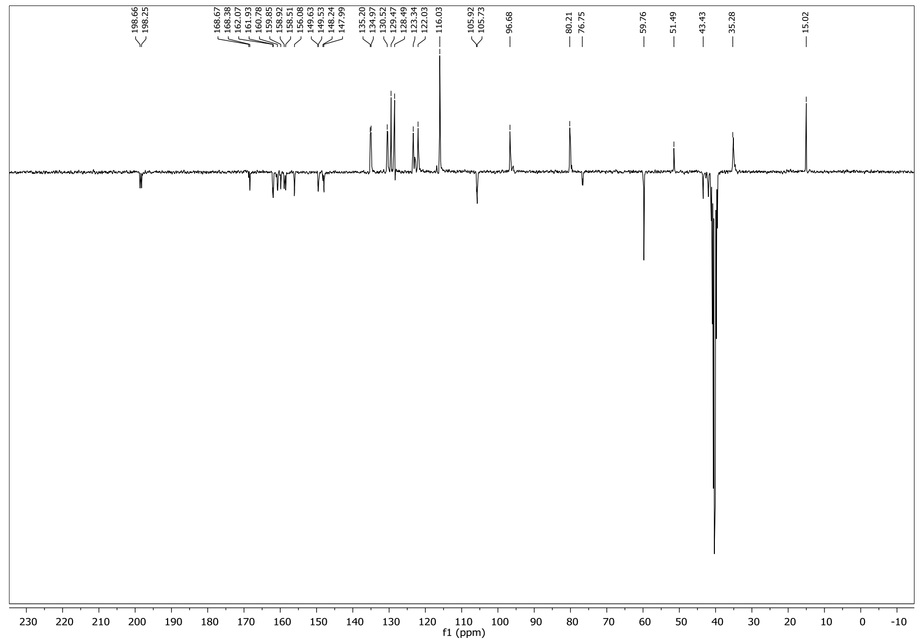


Figure A.49: DEPTQ135 ^{13}C NMR spectrum of **3.2g**

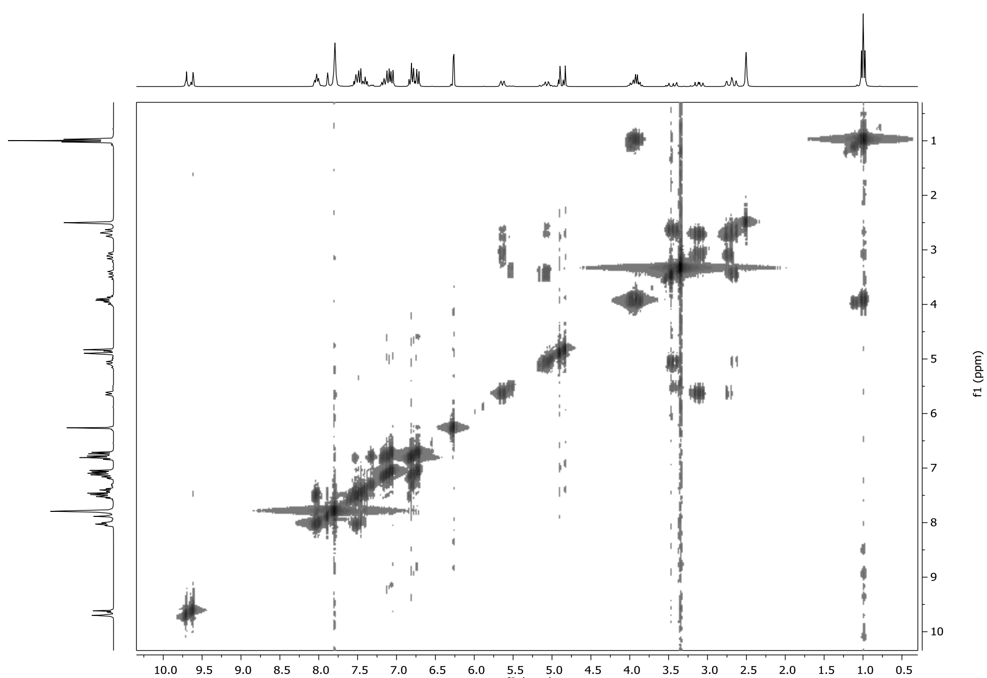


Figure A.50: COSY NMR spectrum of **3.2g**

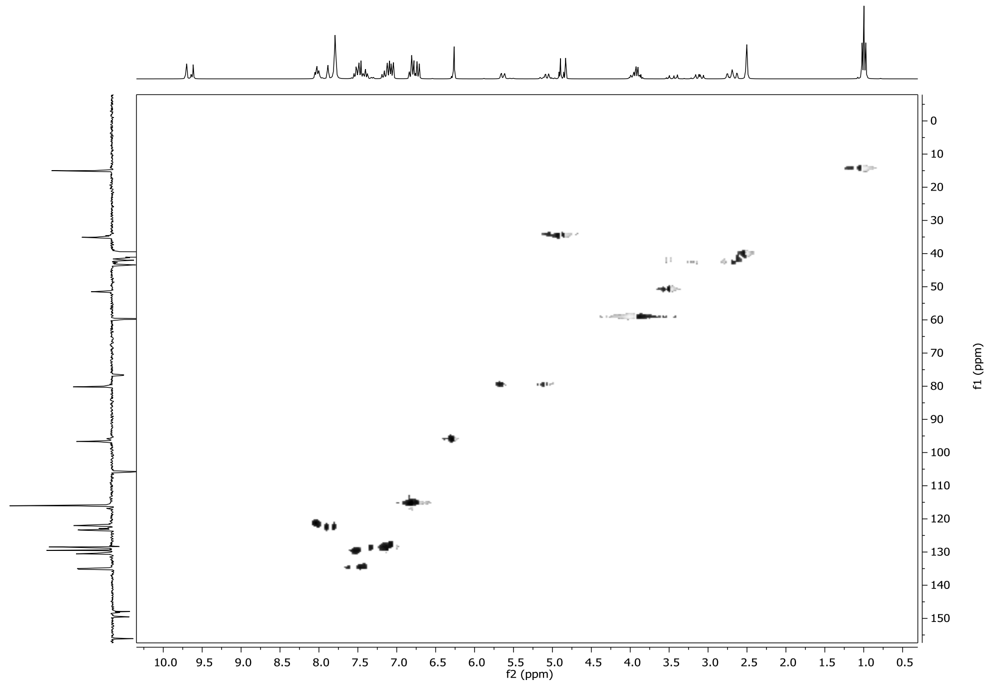


Figure A.51: HSQC NMR spectrum of **3.2g**

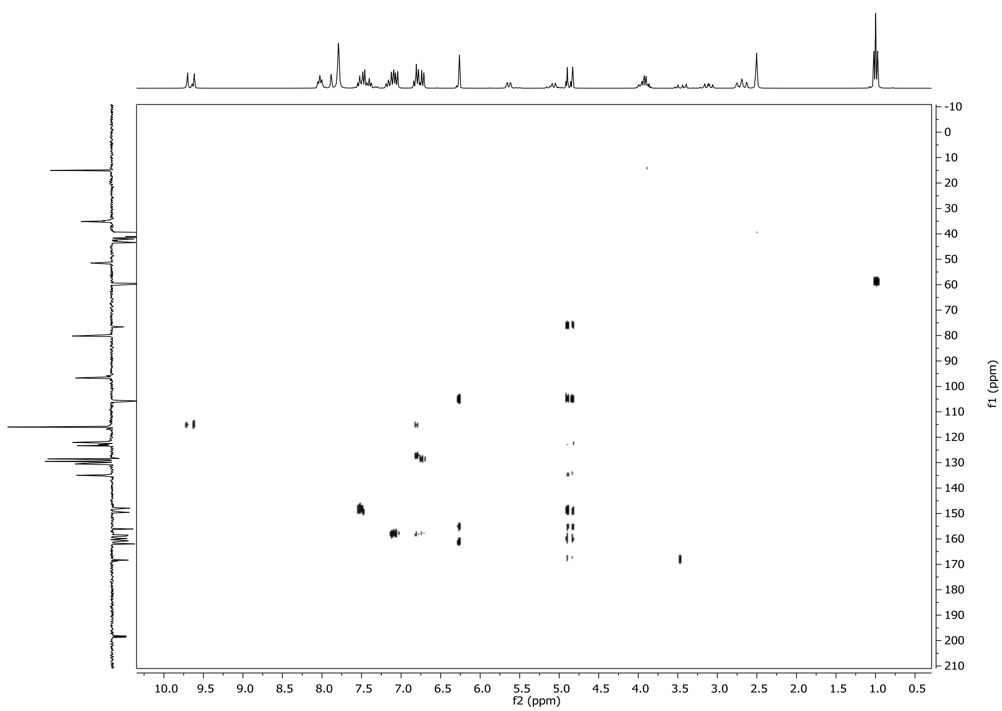


Figure A.52: HMBC NMR spectrum of **3.2g**

A.3 NMR data for Chapter Four

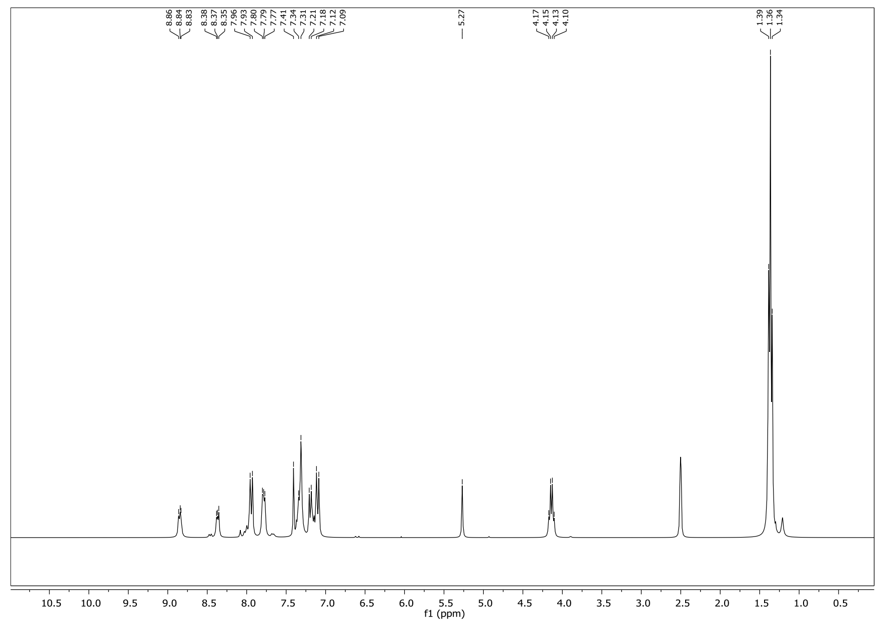


Figure A.53: ^{1}H NMR spectrum of **4.2a**

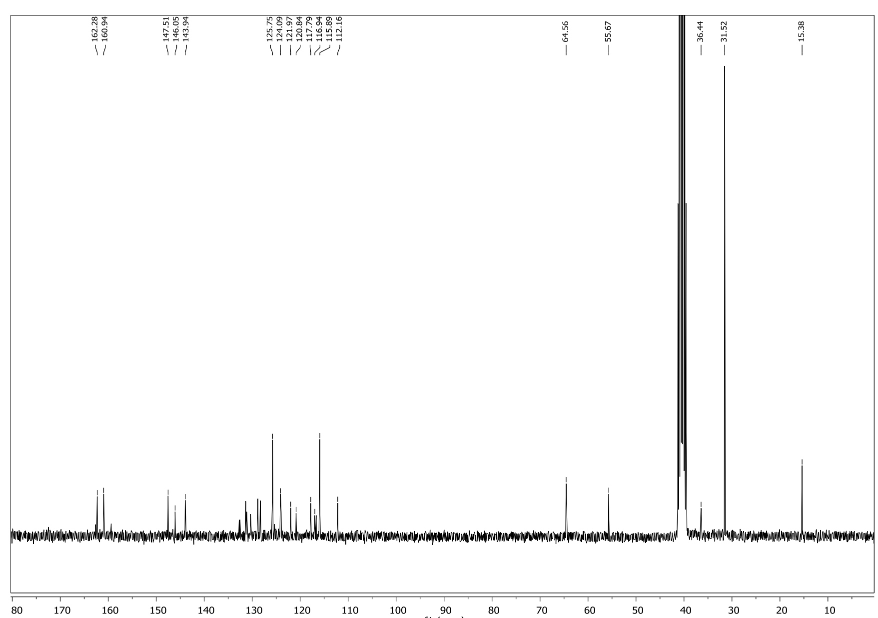


Figure A.54: ^{13}C NMR spectrum of **4.2a**

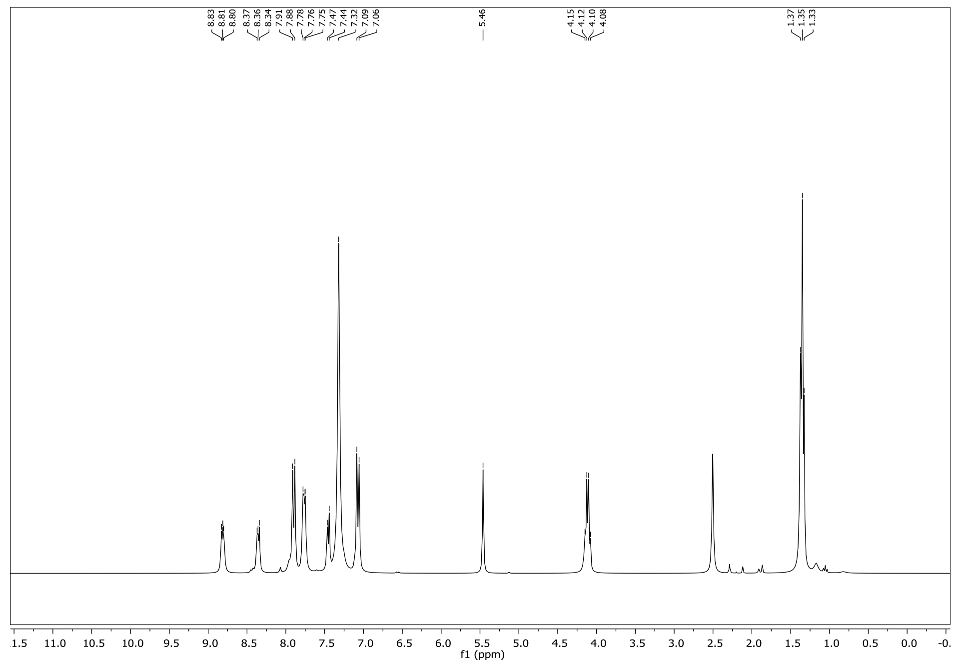


Figure A.55: ^{1}H NMR spectrum of **4.2b**

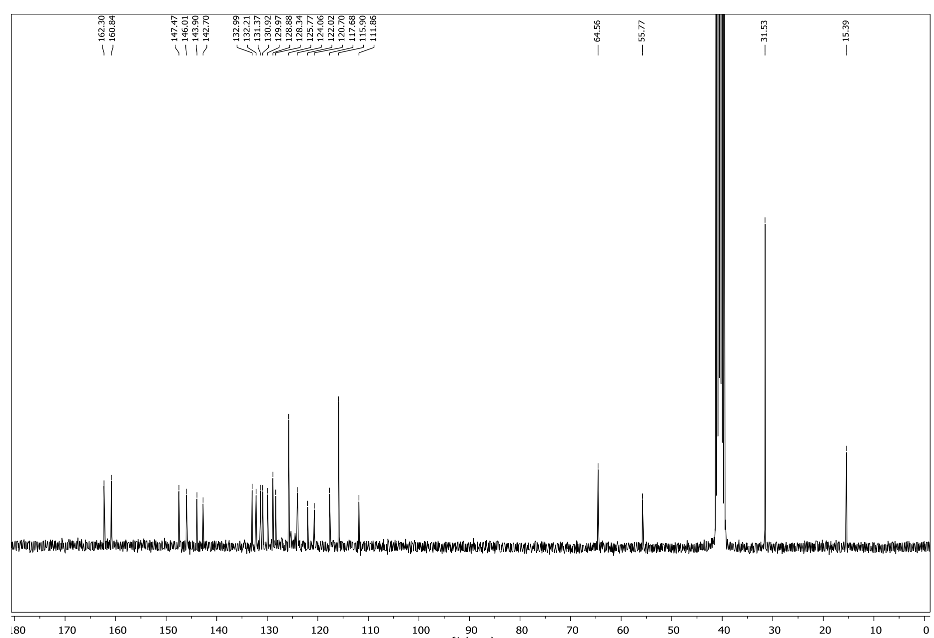


Figure A.56: ^{13}C NMR spectrum of **4.2b**

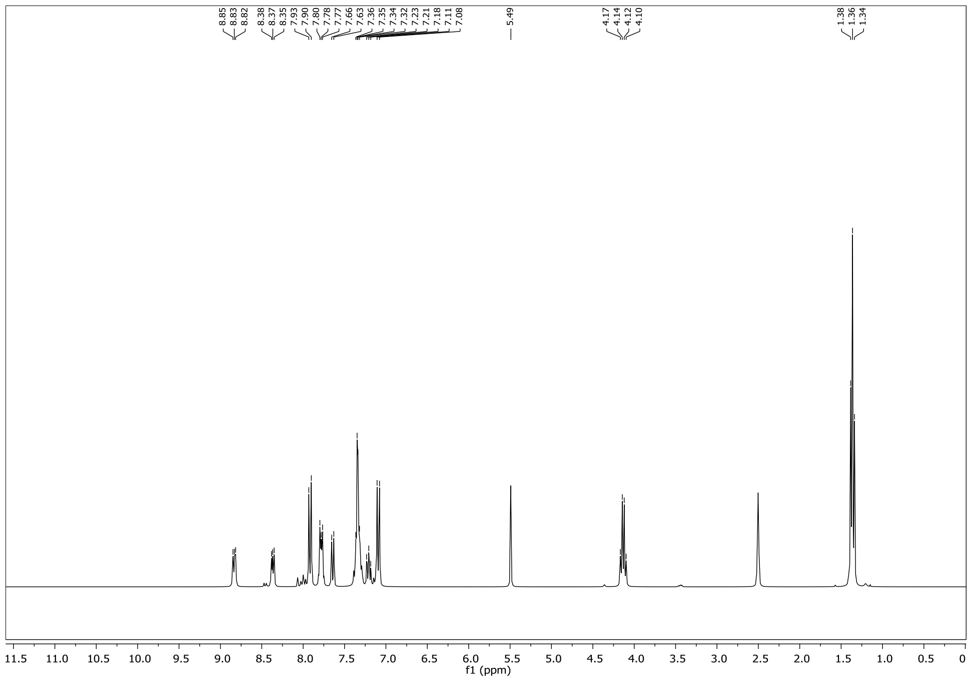


Figure A.57: ^{1}H NMR spectrum of **4.2c**

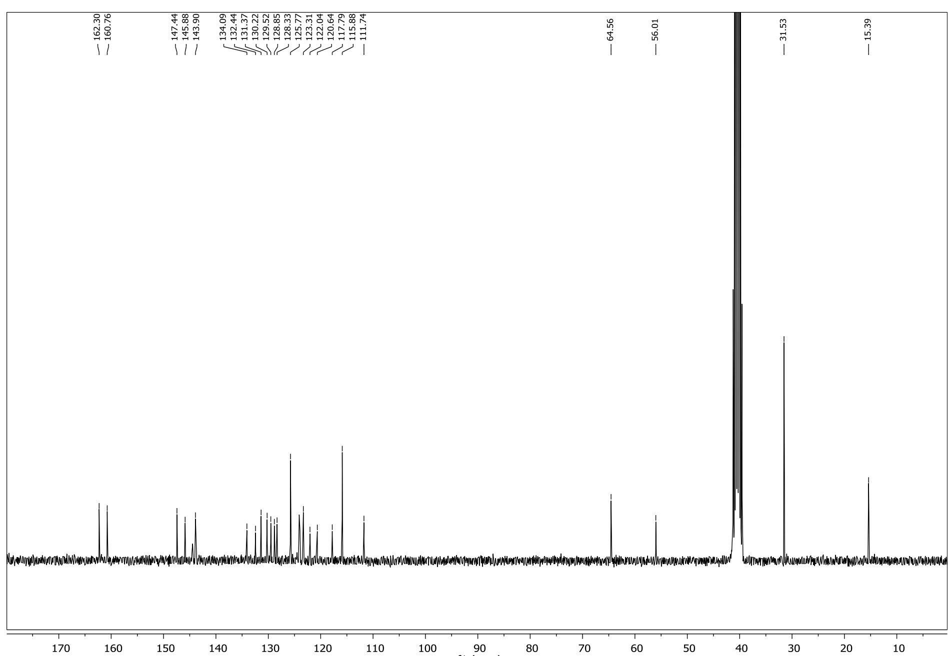


Figure A.58: ^{13}C NMR spectrum of **4.2c**

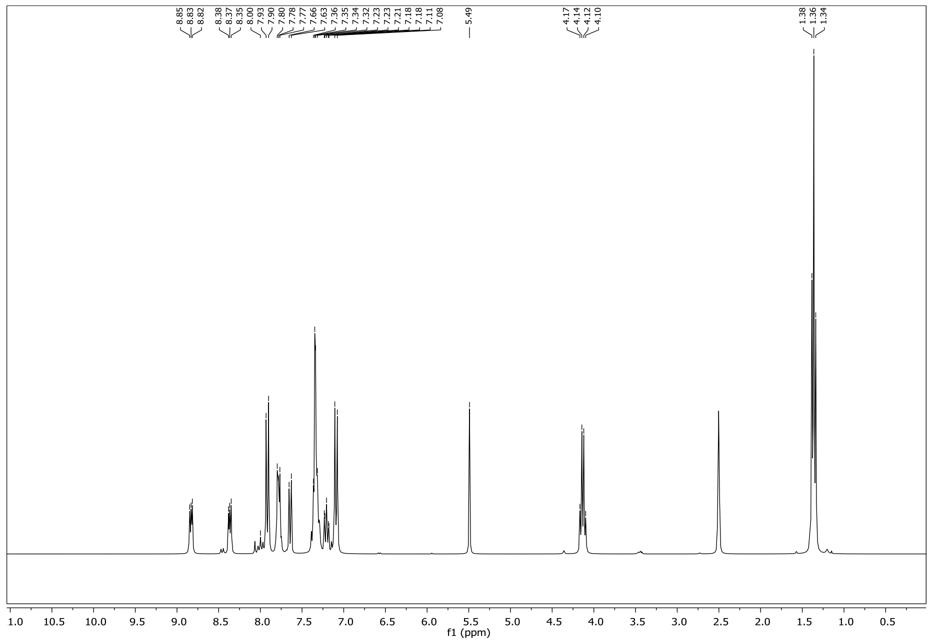


Figure A.59: ^{1}H NMR spectrum of **4.2d**

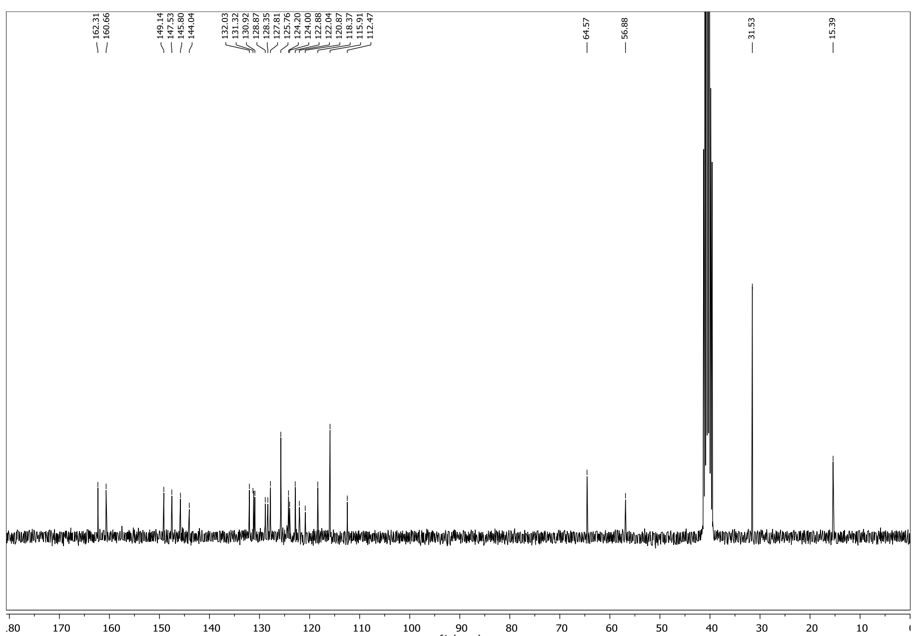


Figure A.60: ^{13}C NMR spectrum of **4.2d**

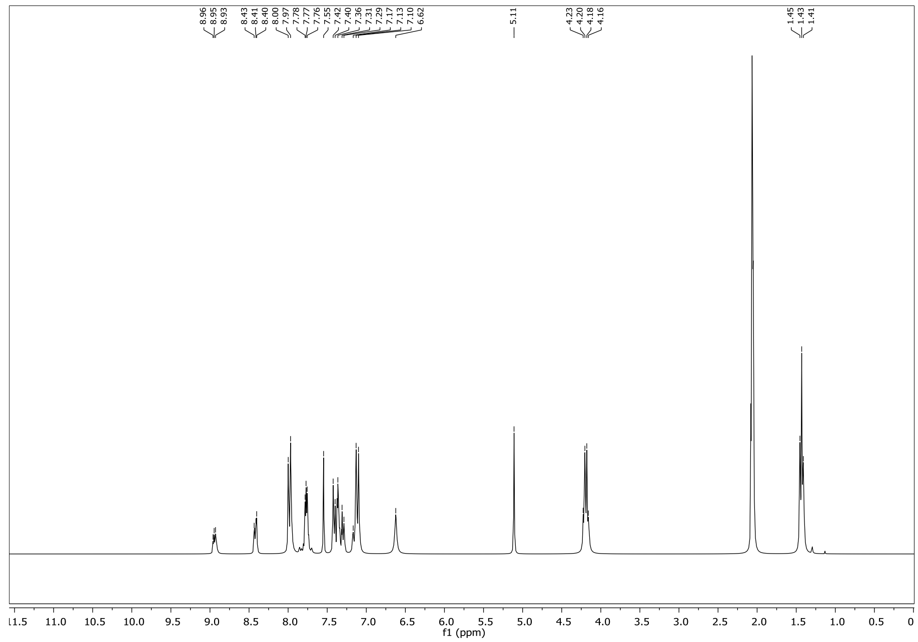


Figure A.61: ^{1}H NMR spectrum of **4.2e**

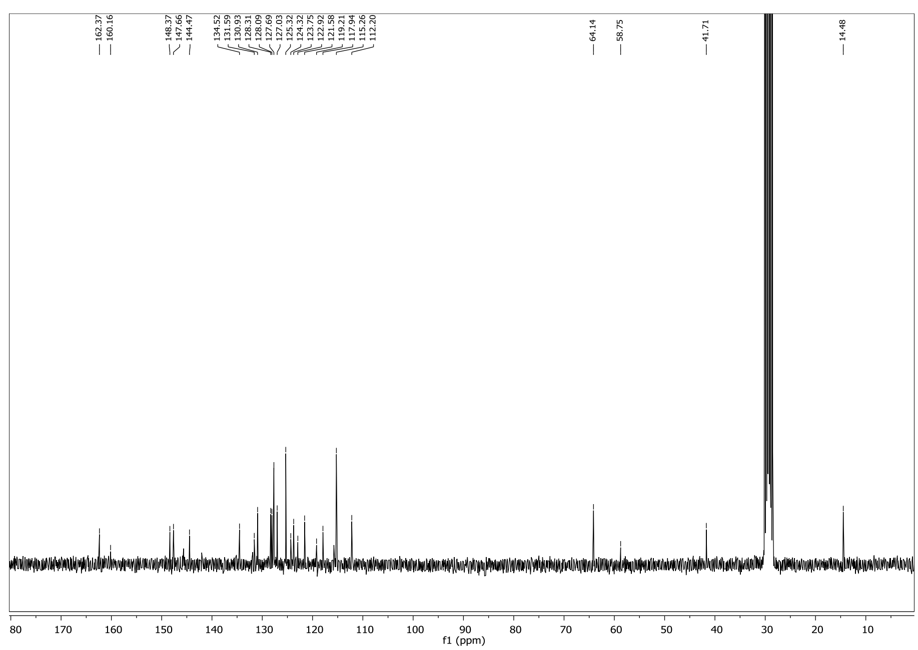


Figure A.62: ^{13}C NMR spectrum of **4.2e**

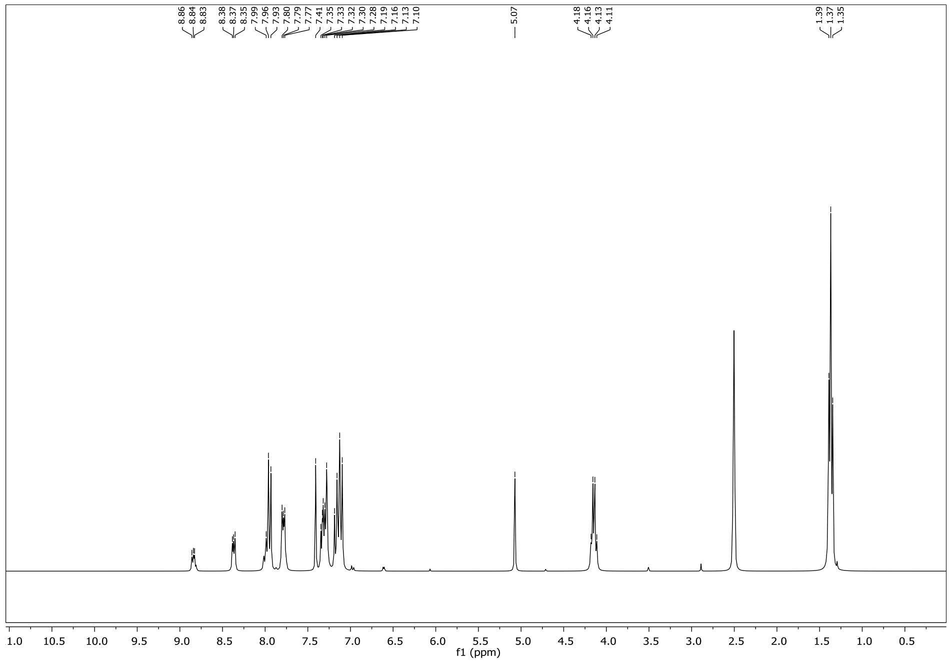


Figure A.63: ^{1}H NMR spectrum of **4.2f**

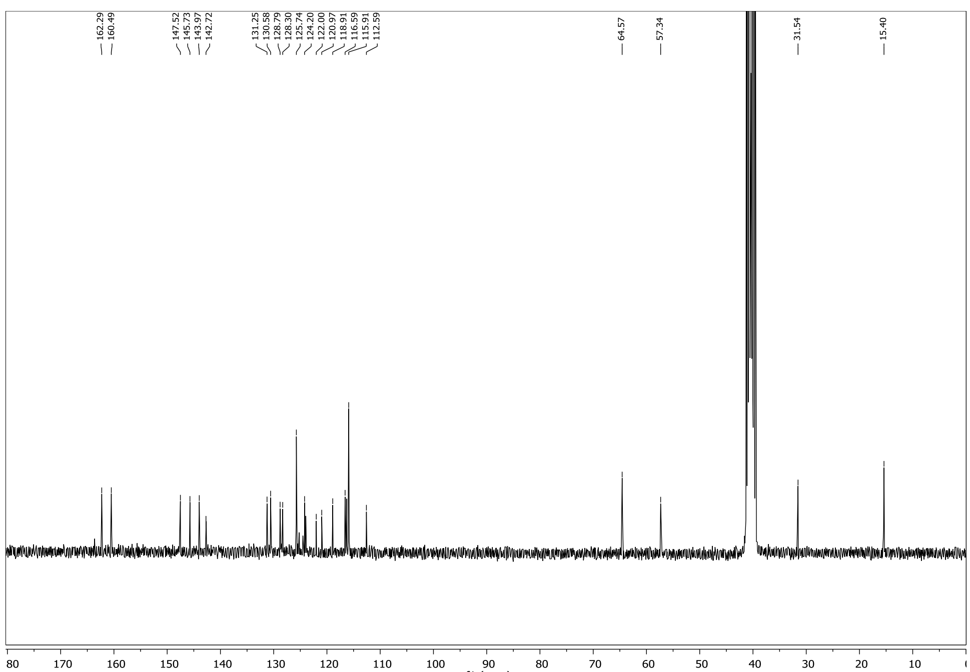


Figure A.64: ^{13}C NMR spectrum of **4.2f**

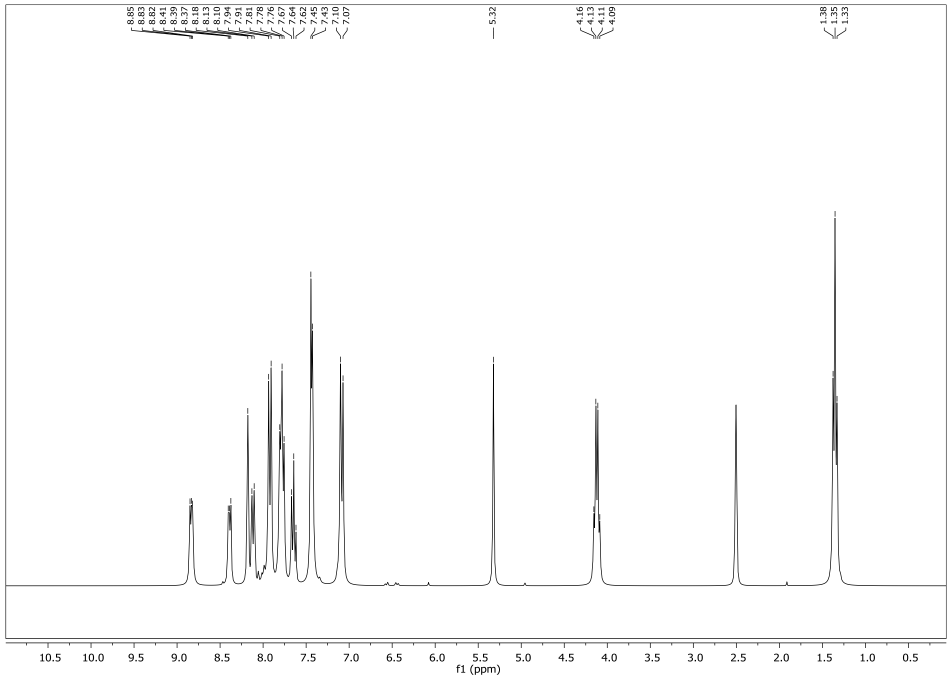


Figure A.65: ^{1}H NMR spectrum of **4.2g**

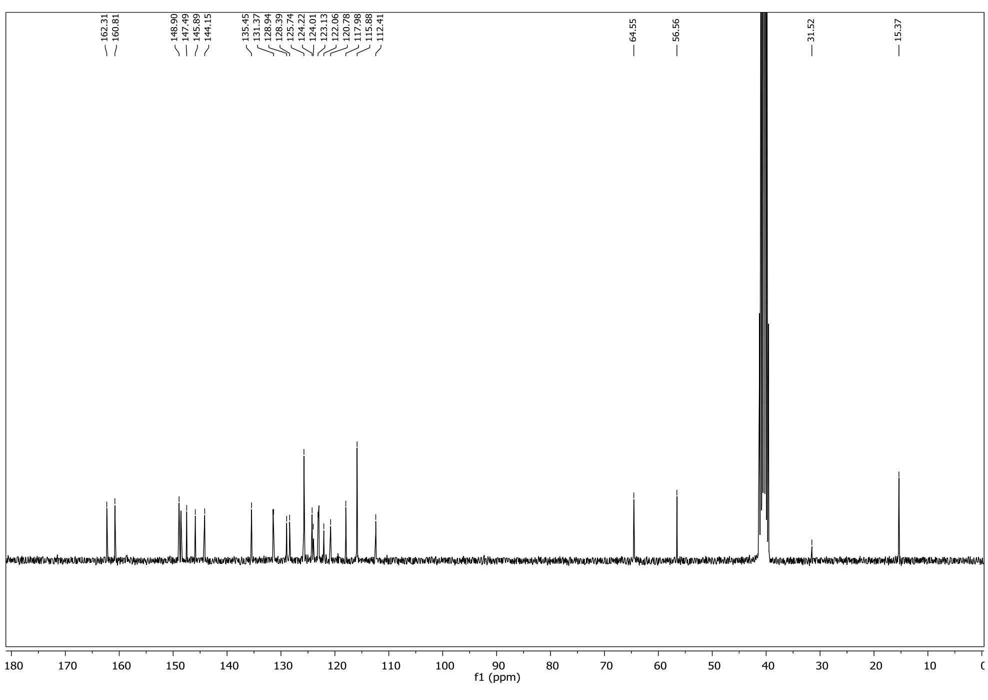


Figure A.66: ^{13}C NMR spectrum of **4.2g**

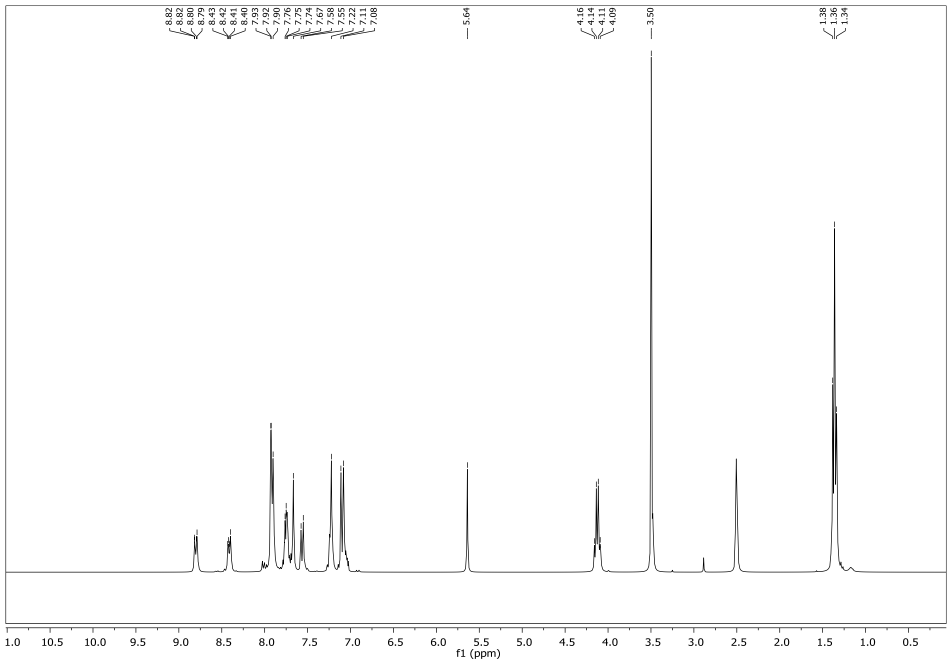


Figure A.67: ^{1}H NMR spectrum of **4.2h**

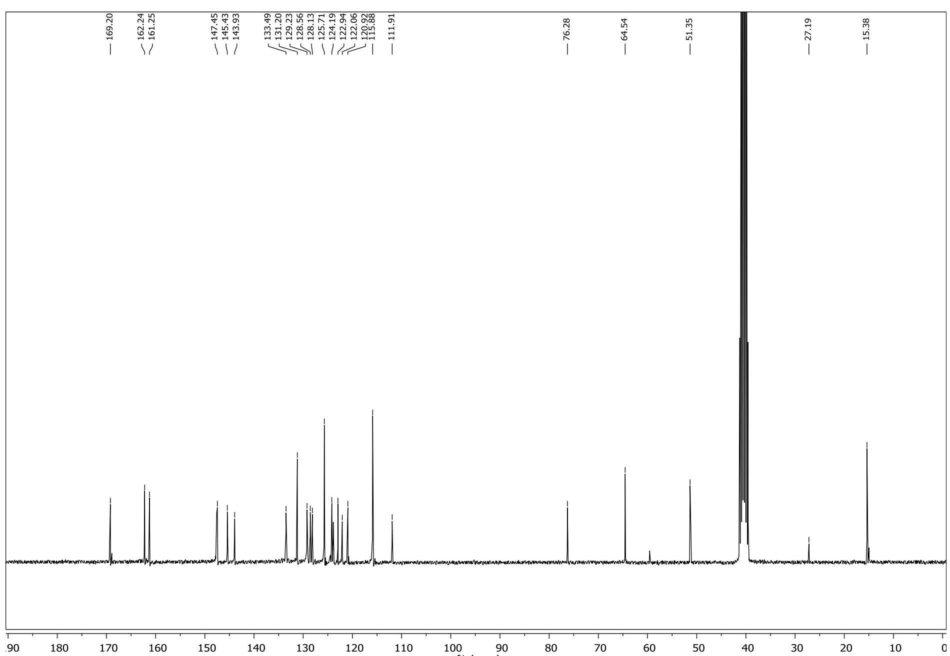


Figure A.68: ^{13}C NMR spectrum of **4.2h**

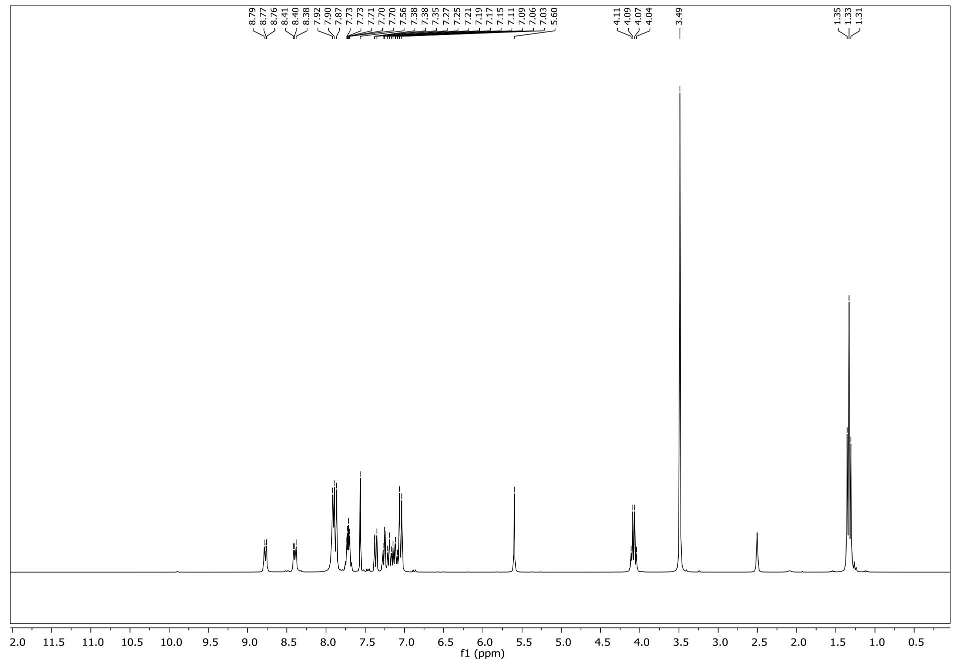


Figure A.69: ^{1}H NMR spectrum of **4.2i**

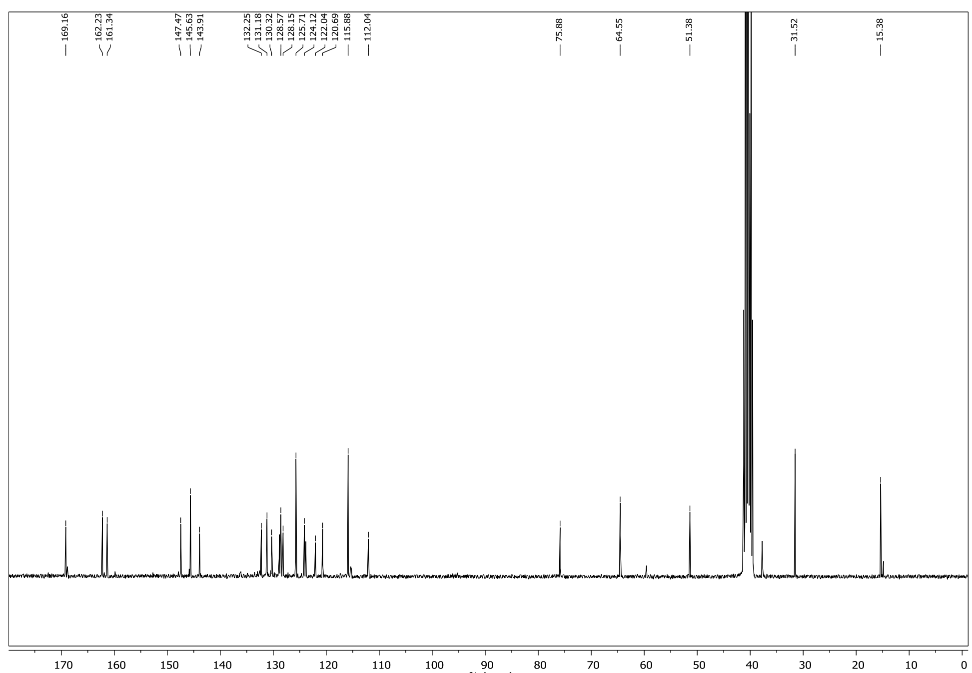


Figure A.70: ^{13}C NMR spectrum of **4.2i**

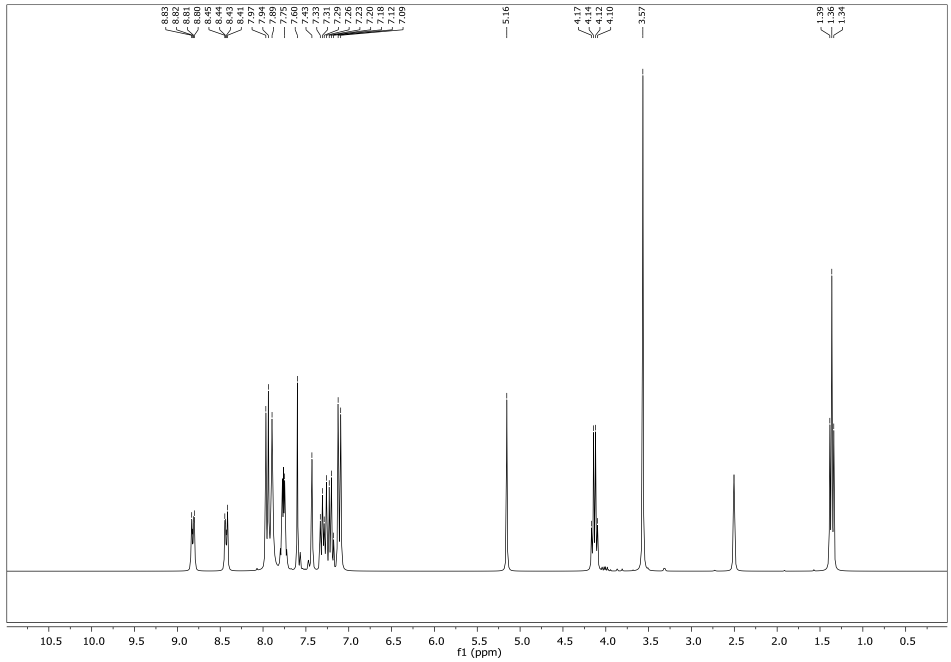


Figure A.71: ^{1}H NMR spectrum of **4.2j**

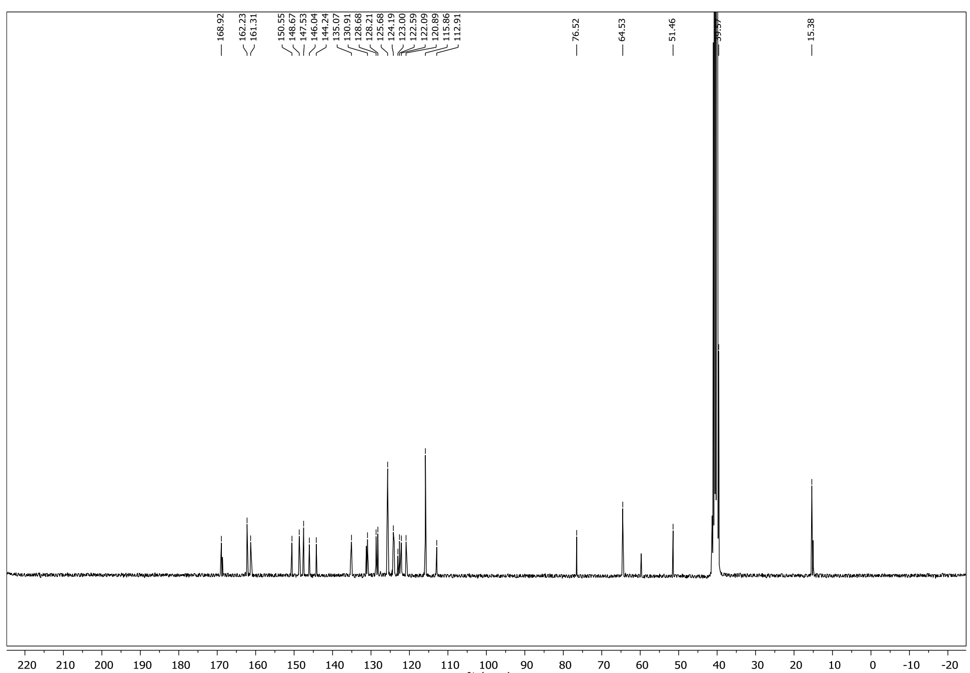


Figure A.72: ^{13}C NMR spectrum of **4.2j**